Exposure to cigarette smoke condensate and its impact on human gingival fibroblast: mechanisms of molecular pathways involved in survival/apoptosis

Mémoire

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Résumé

Notre objectif est d'étudier les effets de la fumée de cigarette (FC) sur les fibroblastes gingivaux humains. Nos travaux démontrent que FC réduit la viabilité cellulaire cellules vivantes par le biais de la voie apoptotique/nécrotique. Une analyse génomique a montré une surexpression significative de plusieurs gènes dont les gènes de *Bax*, récepteurs du *TNF* et caspases; mais une régulation des gènes *Lymphotoxin alpha*, *BCLA1* et *BIRC3*. Une analyse protéique montant une augmentation des protéines Bax et p53 et de la caspase -3 confirmant l'effet nocif du FC biais un mécanisme apoptotique/nécrotique. Une exposition répétée au FC pendant de courtes périodes, favorise la prolifération cellulaire en augmentant l'activité de la télomérase. En conclusion, ces résultats démontrent qu'à hautes doses la FC est toxique mais à faibles doses la FC provoque une surcroissance cellulaire qui pourrait contribuer au développement des maladies parodontales et des caries.

Abstract

The study was to investigate the effects of cigarette (CS) on normal human gingival fibroblasts. Our results showed that continuous exposure to CS led to reduced cell viability through an apoptotic/necrotic pathway. PCR arrays showed significant genes' overexpression such as BCL2-associated X protein (*Bax*), Tumor necrosis factor receptor (*TNF receptors*) and Caspase genes. When fibroblasts were repeatedly exposed to small doses of CS, they showed higher proliferation rates. It is important to note that CS-treated fibroblasts showed a significant increase in telomerase activity when exposed periodically to low level of CS. In conclusion, these results demonstrated that at high dose and longer period, CS was toxic to the gingival cells through an apoptotic pathway. However, at small doses with multiple short time exposures CS promoted cell growth and modulated telomerase activities. This may be contributing to oral disease initiation and development of oral pathologies such as periodontitis or cancer.

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ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
dNTP	deoxyribonucleotide-triphosphate
dscDNA	double strand cDNA
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
g	Gram
H_2O	Water
Kb	Kilo bases
µg	Microgram
min	Minute (s)
ng	Nanogram
nm	Nanometer
OD	Optical density
PCR	Polymerase chain reaction
Q-RT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
RT	Reverse Transcriptase
s	Second (s)
SDS	Sodium Do-decyl Sulfate
TAE	Tris Acetic acid EDTA
TBE	Tris
TE	Tris EDTA
U	Units
UV	Ultraviolet
V	Volt
Bax	BCL2-associated X protein
TNF	Tumor necrosis factor
MMP	Metalloproteinase

Avant-propos (Foreword)

The work with this thesis was performed at the "La Faculté de Médecine Dentaire de l'Université Laval ", as a part of the Master degree in Cellular and Molecular Biology program at the Laval University. The work was started in September 2012.

I was fortunate performing my research about a very interesting subject related to the link between cigarette smoke and oral disease that involve gingival fibroblasts. With the help of different people from Dr Rouabhia's research team, I was able to generate two major publications.

1. Long-term exposure of human gingival fibroblasts to cigarette smoke condensate reduces cell growth by modulating Bax, caspase-3 and p53 expression. This study was already published.
2. Repeated exposure to cigarette smoke condensate induced gingival fibroblast proliferation through the activation of cell cycle genes and telomerase activity; this paper is under preparation for submission.

Different coauthors are listed in each publication, thanks to their active implications in my research activities. Coauthors roles were highlighted in the particular foreword of each publication.

First of all, I would like to thank my supervisor, Professor Mahmoud Rouabhia, for giving me the opportunity to complete my master thesis. I have learned many things since I became Dr. Rouabhia's student. He spends very much time instructing me how to perform all needed protocols to move further adequately with my research project. He also significantly contributed explaining to me how to search appropriate literature, how to collect and analyses data, how to write a manuscript. Etc. Thanks Dr. Rouabhia for your availability, your kindness, for the useful comments, remarks and engagement through the learning process of my master thesis. It's hard to find the right words to say to him, so I will simply say thanks.

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Chapter 1:

Introduction

1.1 Introduction

1.1.1 The object of investigation

Oral cancers constitute a significant health concern for a large number of populations worldwide A profound understanding of the early effects of Cigarette smoke (CS) on gingival cells/tissues will likely provide critical insight into the prevention and therapy of CS-related damage and cancer. Oral cancer consistently ranks as one of the top ten cancers worldwide (approximately 5% of cancers in men and 2% in women), with broad differences in geographic distribution (1, 2). Overall, 10.5 adults per 100,000 will develop oral cancer, with greater prevalence in males compared to females (2). The oral cancer induced by cigarette smoke (CS) is the sixth most common malignancy. It is characterized by a reduced survival rate and a high morbidity rate, which have not changed significantly in the past half-century. Most studies examining tobacco-induced carcinogenesis have focused primarily on the mutagenesis of the epithelial cells, the target cells in the most environmentally induced cancers (3). Our Laboratory recently demonstrated that acute exposure to CS led to a significant effect on the barrier and innate immune function of the gingival epithelial cells and tissues (4, 5). However, the CS effect on gingival fibroblasts has yet to be elucidated. The mechanisms of these effects remain largely unknown and, therefore, must be explored. To attain our goals, we were using normal human gingival fibroblasts cells (HGFs) being subjected to either acute (one) or chronic (multiple) exposures to whole CS. Then the non-treated and smoke-treated cells were used for investigating the effect of CS condensate exposure on gingival fibroblast viability/ growth, apoptosis and telomere activity.

1.1.2 Cigarette smoking

The health effects of smoking cigarettes are destructive and in many cases, deadly. Cigarette smoke is a complex mixture of chemicals of thousands of chemicals, (e.g., nicotine, cadmium, phenol, anthracyclic hydrocarbons, nitrosamines, heavy metals, and chemical carcinogens, etc.) (6). Approximately 7000 chemicals, have been identified in cigarettes and cigarette smoke to date, 250 of which are poisonous and 70, carcinogenic and placed them in group 1 (carcinogenic to humans). Science is far from finished in its exploration of the composition of manufactured tobacco products, and the chemical count is still increasing, and that can affect the health of individuals who inhale it (7). Worldwide, tobacco use causes more than 5 million deaths per year, and current trends show that tobacco use will cause more than 8 million deaths annually by 2030 (2). Cigarette smoking is responsible for more than 480,000 deaths per year in the United States, including an estimated 42,000 deaths resulting from secondhand smoke exposure (2). This is about one in five deaths annually or 1,300 deaths every day (2). The diseases associated with smoking include: chronic obstructive pulmonary disease (COPD), asthma, laryngeal, lung, throat, cervical, kidney, coronary heart disease, stroke, abdominal aortic aneurysm, acute myeloid leukemia, esophageal, stomach, cataracts, pneumonia, periodontitis, and bladder, and pancreatic cancers.

In the oral cavity, cigarette smoking is a major risk factor for cigarette smoking is a risk factor for oral cancer, oral mucosal lesions, and periodontal disease (8). Human gingival fibroblasts (HGFs) are the main cellular component of periodontal connective tissues.

1.1.3 Periodontal disease and cigarette smoking

Smoking is one of the most significant risk factors associated with the development of periodontitis. Periodontal disease is defined as being conditions that range from simple gum inflammation to serious disease that results in significant damage to soft tissue and bone that support the teeth. In the worst cases, there was a loss of teeth. It is the leading cause of tooth loss among adults. Periodontal disease is very common affecting about 90% of the worldwide population (8). Experimental evidence accumulated over the last two decades has indicated that cigarette smoking is probably a real risk factor for periodontitis (9) and even promotes its development (1, 10). Smokers have both increased prevalence and more severe extent of periodontal disease, as well as higher incidence of tooth loss, compared to non-smokers. Several studies have associated cigarette smoking and subgingival infection with periodontal pathogens (11). Other studies have investigated the association between smoking and loss of periodontal bone height (12). It is evident that smokers suffer greater bone loss, greater attachment loss, and deeper periodontal pockets than non-smoking patients (13). **Additionally, smoking can lower the chances of success of some treatments** (13, 14). Three common bacteria are involved in periodontal disease. *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, and Prevotellaintermedia*, are all present in higher amounts in smokers than non-smokers, there is a lot of research that indicates the type of bacteria in smokers is more likely to cause periodontal disease. This is due to a higher quantity of bacteria that is present in smokers (15). The contact between cigarette smoke and oral bacteria promotes such oral diseases as periodontitis and oral candidiasis (16).

1.1.4 Effect of cigarette smoke on oral cavity

There are several ways that smoking disturbs our oral health that includes lesions in the mouth; the most common being gum disease. Smoking and tobacco products can lead to periodontal disease by affecting the attachment of soft tissue and bone to teeth. It seems that smoking interferes with the normal function of gum tissue cells. Smoking may affect wound healing, making smokers more susceptible to these diseases where weakens the blood flow to the gums. The following oral diseases and conditions are caused by smoking (17, 18).

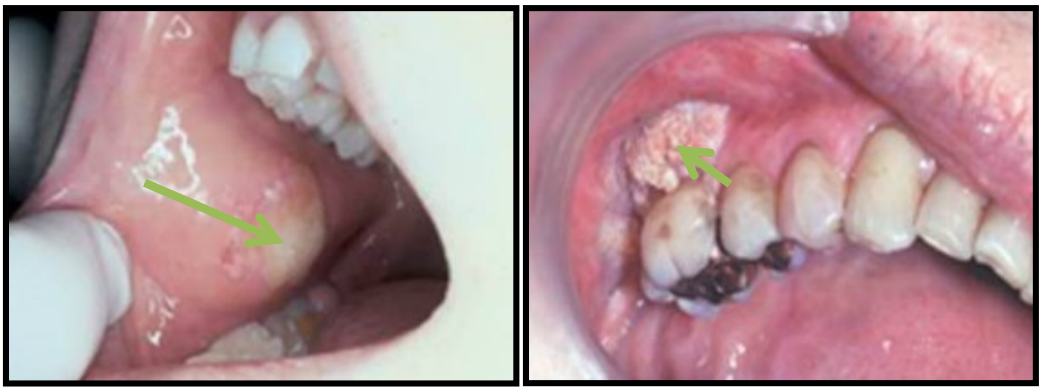

Figure 1-1: showing person suffered of oral cancer that occurs with lump or thick portion of the lip with white patches on the gums (19)

- **Smoker's melanosis** is associated with cigarette and pipe smoking. It refers to brown/dark spots inside the mouth, in the cervical margins of teeth. These spots are caused by tar and other products following cigarette of combustion (20).
- **Coated tongue** is an extrinsic staining, such as dietary substances (e.g., coffee or tea), may contribute to the discoloration. This can be due to the formation of a colored layer composed of mainly bacteria, food particles in the mouth. Tobacco habit promotes plaque accumulation. Treatment consists of mechanical scaling and polishing with mild abrasives. However, the stains will quickly reappear with continued tobacco use (21)

- **Smoker's palate** also known as nicotinic stomatitis is an abnormal manifestation occurring in the hard palate of smokers using pipe, cigar or cigarette smoker. The palate of 45 years old men's exhibiting a diffuse keratotic shape, with irritation to the minor salivary glands. Patient palate can present numerous papules with punctate red centers. This represents the irritated minor salivary glands with inflamed duct orifices (22, 23). Histologic analyses showed hyperkeratosis and acanthosis of the palatal epithelium. There is also inflammation in the connective tissue and minor salivary glands, and squamous metaplasia of the salivary gland ducts (22, 23). Nicotinic stomatitis increases the risk for squamous cell carcinoma different regions including the tonsillar, retromolar, and respiratory tract. Interestingly, Smoker's Palate manifestation can be reversed following tobacco cessation.
- **There are other diseases that affect the oral cavity following smoke.** These include oral thrush, which is a type of fungal infection that occurs in the mouth. Also gingivitis, an inflammatory manifestation being caused by bacterial infection can be promoted by cigarette smoke leading to gum disease (Table 1) (24). There is a link between smoking and the accumulation of dental plaque (biofilms), gingival recession, and deepening of periodontal pockets (25-27). Several biological mechanisms suggested host immunity decreasing with smoking (28). This can be through, harmful effects on the function of neutrophil (29) and reducing blood flow to the gums (30). Cigarette smoking has been associated with impaired healing and less improvement in pocket reduction following simple pocket-reduction surgery (31).
- **Mucosal conditions**. Burns and kiratotik patches are common on the lips at the contact site with the cigarette when used (21). The lesions appear on the surface of the mucosa of the lower and upper lips showing white areas flat or slightly elevated with red striations (21).

Other injuries caused by tobacco smoke may have surface of wrinkles ranging from white, opaque to translucent, and located in an area where snuff. The solution of these pests is to stop smoking. A biopsy may be needed to rule out histological analyses especially if the lesion is associated with compaction, ulceration, erythema and non-resolution within two weeks of stopping tobacco cessation (32).

Table 1-1: Dental and gingival conditions associated with tobacco use (21).

Dental Conditions	Mechanism	Significance	Management[a]
Discolouration	Combustion by-products	Esthetics Plaque trap	Mechanical polishing Whiteners
Abrasion (mild)	Pipe smoking Smokeless tobacco	Dentinal/tooth sensitivity	Desensitization Direct restorations
Abrasion (severe)	Pipe smoking Smokeless tobacco	Pulp exposure Occlusal disharmony	Endodontic/prosthodontics options
Gingival Conditions			
ANUG	Tar/nicotine-induced plaque accumulation Ischemia	Severe gingival destruction	Ultrasonic debridement Antibiotic therapy (rare)
Smoker's melanosis	Stimulation of melanocytes	Must rule out melanoma or systemic conditions	Biopsy may be required to rule out melanoma
Association with gingivitis and periodontitis	Increased calculus and plaque deposits Ischemia	Periodontal destruction	Periodontal therapy (debridement, excisional procedures)
Poor wound healing	Ischemia	Postsurgical care Osteitis	Regenerative periodontal techniques contraindicated

[a] All treatment should coincide with the cessation of the tobacco habit. This Table groups multiple observations made in smokers and there consequences on the oral health. ANUG, acute necrotizing ulcerative gingivitis.

1.1.5 Cigarette smoking and oral cancer

Cancer is a disease in which the normal body cells transform to malignant cells, as a result of changes or mutations in the normal cell genome resulting in uncontrolled cell proliferation. These changes (mutations) produce proteins that disrupt the balance between normal regulatory processes

(e.g. cell cycle) and programmed cell death (apoptosis) leading to cell overgrowth. The mutations are heritable and allow the cancer cells to spread and invade other tissues and organs (metastasis), which can be fatal. Almost 90% of cancer-related deaths are due to metastasis. Although cancer comprises at least 100 different types, and each is classified by the type of cell that is initially affected, all share one important characteristic: the abnormal, uncontrolled cell growth (33).

Oral cancer is considered one of the top ten cancers worldwide, with considerable differences in geographic distribution. It is a primary cause of global morbidity and mortality. Squamous cell carcinoma (SCC) accounts for more than 90% of oral malignancies and occurs most frequently in middle-aged to elderly patients who are heavy smokers and drinkers (34). This type of cancer represents approximately 5% of cancers in men and 2% in women (35). In USA, the National Cancer Institute Survey has shown that oral cancer rates have increased approximately 15% from the mid-1970s until recently (2004). This survey reported significant disparities in some population groups, with higher rates of increase in minority men. Overall, 10.5 adults per 100,000 will develop oral cancer, with greater prevalence in males compared to females. Oral cancer in USA is also dependent on the ethnicity since the rates are higher for Hispanic and Black males than for White males. As for worldwide population, oral cancer rates increase with age. In 2010, the statistics worldwide estimated that the oral cavity, pharynx (other than naso-pharynx), and larynx cancers account for 683,000 new cancer cases (5.2% of global cases) and 356,000 deaths (4.4% of global cancer deaths). The tongue, lip and floor of the mouth constitute the three most common sites for SCC development. SCC can develop from precancerous lesions (leukoplakia and erythroplakia) or even from the normal epithelium. Oral cancer such as squamous cell carcinoma (OSCC) often develops after the age of 50, with the highest peak in the sixth decade of life (36, 37). The exposure to exogenous carcinogens such as tobacco smoke and alcohol represents the primary risk factor for

oral cancer. Studies have shown that oral cancers are highly linked with alcohol consumption and cigarette smoking with an 80% of cases of oral cancers (38). A large number (3 to 6%) of oral cancers is associated with leukoplakia lesions, with an increasing frequency with longer follow-up periods (39, 40). Genetic susceptibility (predisposition) to OSCC is also a major risk factor especially in young patients. This is linked to the efficiencies of carcinogens metabolism, DNA repair, and cell cycle control, alone or in combination (41).

1.1.6 Effect of cigarette smoke and human gingival tissue

Upon entrance into the oral cavity, cigarette smoke reaches the oral mucosa where epithelial cells and fibroblasts interact. Cigarette smoke can have harmful effects on epithelial cells and fibroblasts.

1.1.6.1 Effect of cigarette smoke on gingival epithelial cells.

In the oral cavity, the epithelium forms the first line of defense against toxic agents and bacteria such as periodontopathogens (42). In addition to its function as a protective physicochemical barrier to the outside environment, oral epithelium has a number of metabolic and immunological roles, including fluid and ion transport regulation, mucus production/elimination and participation in innate and adaptive immunity, as well as the modulation of inflammation, cell migration and repair processes (43). These functions are essential to maintaining mucosal homeostasis. Despite the fact that human oral epithelial cells are the first cell type to be exposed to cigarette smoke, few studies have addressed the effect of cigarette smoke on these cells and the possible initiation and development of oral diseases that include cancer and periodontitis. In a recent study, our team reported that exposure to whole cigarette smoke markedly inhibits epithelial cell growth through an apoptosis/necrosis pathway that involves Bax and Bcl-xL proteins and caspase-3 activity. Cigarette smoke also disrupts epithelial cell migration, which may negatively affect periodontal

wound healing (4). It has also been reported that nicotine, one of the cigarette smoke constituents increased the secretion of IL-8 from human gingival epithelial cells (44). Also, cigarette smoke was shown to be able increasing the expression of matrix metalloproteinase (MMP)-2, MMP-9 and MMP-28 by human keratinocytes (45).

1.1.6.2 Effect of cigarette smoke on gingival fibroblasts

Fibroblasts are the predominant cell type inhabiting the gingival connective tissue, play a critical role in gingival tissue structures, extracellular matrix synthesis (26, 46) and tissue repair (27) (28). Available studies showed that exposure of the gingival mucosa to cigarette smoke could have a significant effect on fibroblast function and consequently that of gingival tissue. Indeed, nicotine and cigarette smoke condensate were reported to inhibit fibroblast proliferation and altered their capacity producing proteolytic enzymes (29, 30). Moreover, cigarette smoke extract was found to impair the wound healing process by inhibiting fibroblast recruitment and proliferation. It has been reported that cigarette smoke modulates pulmonary fibroblast-mediated contraction through possible enlarged air spaces that develop in the injuries associated with pulmonary diseases (31). Altogether, these data suggested a significant harmful effect of cigarette smoke on gingival fibroblast. However, critical investigation still to be performed to shed light on the direct effect of cigarette smoke on gingival fibroblasts.

1.2 Hypotheses:

Oral cancer induced by cigarette smoke (CS) is the sixth most common malignancy. It is characterized by a reduced survival rate and a high morbidity rate, which have not changed significantly in the past half-century. Gaining a better understanding of the link between CS and oral cancer is, therefore, critical. Most studies have used the epithelial cells as a model for examining tobacco-induced carcinogenesis. Previous work from our group clearly demonstrated

that acute exposure to CS significantly affected the barrier and innate immune function of gingival epithelial cells and tissues. Epithelial cells are in contact with gingival fibroblasts. It's known that the fibroblast-epithelial cells interaction is very important for tissue homeostasis, and the impairment of this interaction may lead to oral cancer development. Fibroblasts can secrete many growth factors such as TGFb and other cytokines that can influence the function of epithelial cells. Deregulation of the fibroblast functions would have consequences on the function of epithelial cells. However, the mechanisms related to CS effects on gingival fibroblasts remain largely unknown.

1.3 Objectives

The general objective of this study was to investigate the harmful effect of Cigarette smoke condensate (CSC) on human gingival fibroblast (HGF).

Specific objectives:

1. Investigate the effect of a long-term chronic exposure to CSC on gingival fibroblast viability and apoptosis
2. Investigate the effect of a short but repeated exposure to CSC on gingival fibroblast behavior

Chapter 2

Article 1

Article – 1

Long-term exposure of human gingival fibroblasts to cigarette smoke condensate reduces cell growth by modulating Bax, caspase-3 and p53 expression.

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Foreword

This article includes an introduction, an experimental protocol, results, discussion and conclusion. This publication was obtained thanks to the contribution of the following authors: **Alamri A**, Semlali A, Jacques E, Alanazi M, Zakrzewski A, Chmielewski W, Rouabhia M.

Alamri A conducted all the experiments with the help of Semlali A, Jacques E and Rouabhia M.

Alamri A, Semlali A, Jacques E, Chmielewski W and Rouabhia M, analyzed and interpreted the data.

Alamri A, Semlali A, Jacques E drafted the first version of the manuscript.

Mahmoud Rouabhia completed the manuscript with the help of Alanazi M, Zakrzewski A and Chmielewski W.

All authors read and approved the published manuscript.

2.1 Abstract

BACKGROUND AND OBJECTIVE:

Smoking cigarettes increases the risk of oral tissue damage leading to periodontal disease. Gingival fibroblasts, the predominant cell type inhabiting gingival connective tissue, play a critical role in remodeling and maintaining gingival structure. The objective of this study was to investigate the effect of long-term exposure to cigarette smoke on human gingival fibroblast survival/apoptosis and the molecular pathways involved in these cell responses.

MATERIAL AND METHODS:

Human gingival fibroblasts were extracted from healthy non-smokers and cultured in the presence of cigarette smoke condensate (CSC). At the end of each time point, cell growth was evaluated by means of MTT assay. Apoptotic and necrotic gene's expression was investigated by polymerase chain reaction array and by annexin V/propidium iodide staining and cell cycle assays. Western blot was used to investigate Bax and p53 proteins. These tests were supported by caspase 3 activity analyses.

RESULTS:

High levels of CSC decreased cell growth and deregulated cell cycle progression by increasing the G_0 /G_1 and reducing the S and G_2 /M phases of the gingival fibroblasts. Polymerase chain reaction arrays revealed the activation of several apoptotic genes by CSC, including TNF receptors, caspases, Bax and p53. This was supported by increases in the Bax and p53 protein levels as well as by an elevated activity of caspase-3 in the CSC-exposed cells. Immunofluorescence staining demonstrated that both Bax and caspase-3 displayed a cytosolic and mitochondrial distribution in the CSC-exposed gingival fibroblasts, compared to controls. The damaging effect of CSC on

gingival fibroblast growth was also supported by the decrease in interleukin 6 and 8 secretion by the gingival fibroblasts.

CONCLUSION:

These results suggest that CSC may contribute to deregulating fibroblast functions. This can compromise fibroblast-epithelial cell interactions, which ultimately increases the risk of gingival tissue damage and the onset of periodontitis.

***Keywords*:** Cigarette smoke, human cells, apoptosis, Bax, caspase, PCR Arrays, p53, IL-6, IL-8.

2.2 Introduction

The periodontium consists of a group of tissues that surround and support each tooth as well as retain it in the mandibular and maxillary bones. For these reasons, it is important to maintain healthy periodontium (1). Periodontal tissue health depends on the normal functions of periodontal cells. The attachment, migration, growth, and differentiation of periodontal cells are key steps in maintaining the functionality of periodontal tissues. Unfortunately, periodontal tissues are subjected to multiple insults which can lead to tissue dysfunction in the form of periodontal diseases (2).

Periodontitis is a complex chronic disease that leads to the destruction of tooth-supporting tissues, including alveolar bone, eventually resulting in tooth loss. The disease is believed to develop as a result of the host-mediated inflammatory response to pathogenic microflora residing in periodontal pockets (3). Among the different cell types in the periodontium that may be involved in the host immune response to periodontitis are gingival fibroblasts. As the main cellular component of periodontal connective tissue, these cells play a major role in periodontal health (4). Gingival fibroblasts are active in the inflammatory response by secreting inflammatory cytokines, such as IL-6 and IL-8, as well as inflammatory chemical mediators, such as PGE_2, in response to stimuli that include periodontopathic bacteria (5,6). Aside from the presence of periodontopathic bacteria in the oral cavity, several other factors actively contribute to the onset and progression of periodontitis, including such environmental factors as smoking (7).

It is well recognized that cigarette smoke can alter cell function and promote periodontal disease development and severity (7). Cigarette smoking is a risk factor in both the incidence and the progression of periodontal disease (8). Smokers exhibit significant damage to the alveolar bone which may augment tooth loss (9). Periodontitis severity has been shown to increase with smoking

intensity and duration (10). Furthermore, cigarette smoke also reduces the host response to periodontopathic bacteria, resulting in a more aggressive periodontal breakdown (11). Early studies have reported impaired phagocytosis by *in situ* smoke-exposed oral polymorphonuclear neutrophils (12). Smoking also appears to inhibit host defenses against microbial infection while promoting inflammatory reactions (13). Indeed, smokers are susceptible to colonization by *P. gingivalis*, a causative agent of periodontitis (14). Upon entering the oral cavity, cigarette smoke reaches the oral mucosa where epithelial cells and fibroblasts interact and maintain tissue integrity and function (15). Gingival fibroblasts, the predominant cell type inhabiting gingival connective tissue, play a critical role in remodeling and maintaining gingival structure and extracellular matrix (16,17) and are key players in tissue repair and wound healing through their adhesion, migration, growth, and differentiation, as well as through the production of extracellular matrix (16,17). Exposure of the gingival mucosa to cigarette smoke may have a significant impact on fibroblast function and consequently, that of gingival tissue. Cigarette smoke products, such as nicotine, act on periodontal cells by promoting the production of inflammatory cytokines (IL-1β and TNF-α) and inducing changes in cell cycle and differentiation marker values (18,19). Furthermore, nicotine has been shown to inhibit the growth of human periodontal ligament fibroblasts through apoptotic mechanisms (20). Tissue damage, as a result of smoking, cannot only impact the integrity of gingival tissue, but can also potentiate inflammatory responses as well as setting up an optimal environment for bacterial growth (21). Based on these data, we investigated the effects of cigarette smoke condensate (CSC) on normal human gingival fibroblast viability/growth, apoptotic process, and expression of specific genes followed by the production of proteins by these fibroblasts.

2.3 Materials and Methods

2.3.1 Preparation of cigarette smoke condensate. 1R3F cigarettes were purchased from the Kentucky Tobacco Research & Development Center (Orlando, FL, USA) and were used to prepare the cigarette smoke condensate solution as follows: Each cigarette was placed into one end of a silicone tube linked to an Erlenmeyer containing 200 ml of 0.09% sodium chloride. On the other end, a second silicone tube linked to a standard vacuum. The cigarette was lit and attached to a cigarette holder. The smoke was extracted by applying a vacuum to draw the smoke directly into the 0.09% sodium chloride solution. The process was repeated for a total of ten cigarettes. The cigarette smoke condensate (CSC) solution was then sterilized by filtration through a 0.22-μm filter and subsequently stored at 4°C until use.

2.3.2 Gingival fibroblast cell culture. Gingival fibroblasts were isolated from gingival mucosa biopsies taken from healthy non-smoker subjects. Written informed consent was obtained from each subject. The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2. The medium was changed three times a week. When the cells reached 90% confluence, they were detached with 0.05% trypsin-EDTA, seeded in 6-well plates at 4 x 10^5 cells/well, and incubated for 24 h to allow for proper cell adhesion. Various concentrations (0, 10, 30, or 50%) of CSC were added to the culture medium, after which time the cells were cultured for 5, 7, 10, and 14 days. Each medium with and without CSC were refreshed every two days.

2.3.3 Cell viability/growth assay. Following fibroblast culture in the presence or absence of CSC at various concentrations, fibroblast shape and density was determined by means of inverted microscopy observations and photos. Cell viability/growth was assessed by an MTT assay. Briefly, a stock solution (5 mg/ml) of MTT was prepared in PBS and added to each culture well at a final concentration of 1% (v/v). The fibroblast cultures were then incubated for 4 h at 37°C with the MTT. The supernatant was then removed, 2 ml 0.04 *N* HCl in isopropanol were added to each culture plate, and incubation was extended for another 15 min. Finally, 200 µl (in triplicate) of the reaction mixture was transferred to the wells of a 96-well flat-bottom plate, with absorbance measured at 550 nm by means of an enzyme-linked immunosorbent assay (ELISA) reader (X-Mark microplate spectrophotometer, BioRad Laboratories, Mississauga, ON, Canada). Baseline optical density accounting for the test plate was subtracted from each experiment. Tests of this assay repeatedly showed a linear relationship between cell number and optical density readings.

2.3.4 Apoptosis assay. An apoptosis/survival assay was performed on fibroblasts in contact with CSC for various periods. For this purpose, we used the Annexin V–fluorescein isothiocyanate detection kit (BD Bioscience, Mississauga, ON, Canada). Fibroblasts were detached from the culture plates with trypsin-EDTA and washed twice with culture medium. The resulting fibroblast pellets were suspended in binding buffer and incubated with Annexin V–fluorescein isothiocyanate and propidium iodide (PI), according to the manufacturer's instructions. Stained cell suspensions were analyzed using the Beckman Coulter Epics Elite ESP cytometer. The cells were categorized as follows: viable cells refer to Annexin V and PI non-stained cells and apoptotic cells refer to the Annexin V-stained but PI non-stained cells.

2.3.5 Propidium iodide staining and flow cytometry analysis of cell cycle distribution following contact with cigarette smoke condensate. Gingival fibroblasts were seeded in a 6-well plate and left for 24 h to allow for cell adhesion, after which time the fibroblasts were subjected or not to CSC treatment (10, 30, or 50%) during 5 days, with medium changing every 48 h. The CSC-treated cells were harvested and 1 x 10^6 cells/ml were fixed with cold 70% ethanol for 1 h on ice. Following multiple washes in cold PBS, the cells were treated with RNase (10 μg/ml) at 37ºC for 1 h, after which time ropidium iodide (PI; 50 μg/ml) was added prior to analysis. Analyses were performed with an Epics® Elite ESP flow cytometer (Beckman Coulter, Miami, FL, USA). The single cell population was gated using pulse width vs. pulse area to exclude clumps and doublets and the scatter plot was used to exclude any obvious debris. The PI was detected using a FL4 channel vs. a cell count histogram plot.

2.3.6 Caspase-3 activity assay. Caspase-3 activity was measured on fibroblasts in contact or not with CSC using the caspase-3/CPP32 colorimetric assay kit (BioVision, Inc. Milpitas, CA, USA) according to the manufacturer's instructions. Briefly, 2 x 10^6 cells were suspended in lysis buffer. Total protein extracts (100 μg) were combined with reaction buffer and 200 μM of DEVD-pNA substrate at 37°C for 1 h. Thereafter, samples were read at 405 nm on a microplate reader and the percentage of activity was determined by comparing each sample with the optical density obtained with a cell extract prepared from cells cultured in the absence of CSC.

2.3.7 Visualization by immunofluorescence of casapase-3 and Bax in the cytosol and mitochondria of cells exposed to CSC. Cells were seeded on coverslips and allowed to reach half confluence. After exposing the cells to CSC for 5 days, the mitochondria were labeled with MitoTracker™ CMXRos-H2 (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's

instructions. Briefly, on the final incubation day with CSC, MitoTracker was diluted to a final concentration of 1 μM in serum-free medium and was added to the cells for 45 min at 37ºC in a 5% CO_2 humidified incubator. The cells were then washed in PBS, fixed, and permeabilized with methanol for 15 min at -20ºC. Coverslips were blocked with rat serum and stained with either a mouse anti-human Bax or a mouse anti-human caspase-3 (10 μg/ml; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) in 0.1% saponin for 1 h and with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes (1:500 final) for 1 h in the dark. Cell nuclei were then stained with 1 mg/mL of Hoechst (Invitrogen, Carlsbad, CA, USA) for 10 min. The slides were subsequently mounted with the PBS/glycerol/gelatin and were visualized under a Zeiss Apotome® microscope with a 63 x /1.4 NA lens and AxioVision 4.8.2 scanning software (Carl Zeiss, Gottingen, Germany).

2.3.8 Real-time PCR array. Total RNA was extracted from cells cultured in the presence of either 0 or 30% CSC for 5 days by means of the RNAspin mini kit (GE, Mississauga, ON, Canada) according to the manufacturer's protocol. RNA concentrations and quality were determined by the Experion automated electrophoresis station from Biorad (Mississauga, ON, Canada). RNA quality was optimal in all samples with 28S/18S rRNA ratios above 1.8. Real-time PCR arrays were performed by means of the RT^2 Profiler PCR Array System from SuperArray Bioscience (Frederick, MD, USA) according to the manufacturer's instructions. Briefly, total RNA (1000 ng) was used to prepare cDNA with the RT^2 first strand kit (QIAGEN, Germantown, MD, USA). PCR arrays containing 88 gene-specific primers related to apoptosis were amplified. The results were analyzed using the $^{\Delta\Delta}$Ct method and the fold changes between the non-stimulated and 30% CSC-stimulated samples were calculated. Genes were selected if a fold change above 1.3 was observed and if baseline cycle thresholds for this gene were below 30 to eliminate feebly expressed genes.

2.3.9 Western blots. Following exposure to CSC, fibroblasts were resuspended in lysis buffer containing 25 mM of Tris-HCl, pH 8.0, 150 mM of NaCl, 1 mM of EDTA, 10% glycerol, 0.1% SDS and 1% Triton X-100. Proteinase inhibitor PMSF 300 mM was then added to the homogenized samples and protein concentration was determined using the Bradford assay. Equal amounts of total protein (20–30 μg) in reducing sample buffer (containing 2.5% 2-mercaptoethanol) were boiled for 5 min and subsequently migrated using 4% stacking gel followed by 10 or 15% acrylamide SDS-PAGE. The gels were then transferred to PVDF membranes with a Tris-glycine refrigerated transfer buffer containing 15% methanol. The blots were incubated overnight with anti-Bax at 1:200 (Santa Cruz Biotechnology), anti-p53 at 1:500 (R&D Systems, Minneapolis, MN, USA) or anti-β-actin at 1:1000 (Sigma). The resulting membranes were washed and finally incubated with an anti-mouse peroxydase-conjugated antibody (BD Bioscience). Detection was performed thereafter by means of the ECL detection system (EMD Millipore Billerica, MA, USA) according to the manufacturer's instructions. Luminescence was obtained by autoradiography. The modulation of protein production was determined by band scanning using Genetools software from Syngene **(**Frederick, MD, USA).

2.3.10 IL-6 and IL-8 measurement following fibroblast exposure to cigarette smoke condensate. Following exposure to CSC for various culture periods, supernatants were collected from each condition and analyzed by sandwich enzyme-linked immunosorbent assay (ELISA, R&D System). Immediately after, the supernatants were filtered through 0.22-μm filters and used to measure the IL-6 and IL-8 levels. ELISA plates were read at 450 nm and analyzed using a Microplate Reader Model 680 (Bio-Rad, USA). The minimum detectable concentrations were under 0.7 pg/ml for the

IL-6, and 3.5 pg/ml for the IL-8, as reported by the manufacturer. Data were reported as pg/ml per ug total protein in each condition to have useful comparisons.

2.3.11 Statistical analyses. Each experiment was performed at least three times, with experimental values expressed as means ± SD. The statistical significance of the differences between the control (absence of CSC) and the test (presence of CSC) values was determined by one-way ANOVA. *Posteriori* comparisons were conducted using Tukey's method. Normality and variance assumptions were verified by means of the Shapiro-Wilk test and the Brown and Forsythe test, respectively. All of the assumptions were fulfilled. P values were declared significant at ≤ 0.05. Data were analyzed using the SAS version 8.2 statistical package (SAS Institute Inc., Cary, NC, USA).

2.4 Results

2.4.1 Cigarette smoke condensate reduced gingival fibroblast growth. Following continuous exposure to CSC for various culture periods, we observed a reduction in cell density in those cultures put in contact with CSC. This treatment led to altered cell morphology, which went from a small elongated cell shape (control) to a large-sized cell with a faint cytoplasm beginning at 7 days and continuing up to 14 days of culture with CSC (Fig. 1A). To confirm these observations, we performed a quantitative measurement of cell viability/growth using an MTT assay. Exposure to CSC did indeed inhibit human gingival fibroblast growth (Fig. 1B). Cells grown without CSC showed increased optical density between cultures of 5 days (0.89 ± 0.03), 7 days (1.68 ± 0.05, $p = 10^{-17}$), 10 days (2.38 ± 0.07, $p = 10^{-20}$), and 14 days (2.77 ± 0.77, $p = 10^{-17}$) (Fig. 1B). However, cells grown in the presence of CSC showed a decrease in growth over the time course. We also demonstrated that cell growth in the CSC-exposed cultures (10, 30 or 50% CSC) was significantly ($p < 10^{-10}$) lower than that in the non-stimulated controls at the different culture periods (5, 7, 10, and 14 days). The growth rate of the fibroblasts differed depending on the CSC concentration. In the presence of CSC at 10,30, and even 50%, a significant proliferation of the gingival fibroblasts was observed after 5, 7, 10, and 14 days (Fig 1B) ,

2.4.2 Cigarette smoke condensate promoted human gingival fibroblast apoptosis. To determine whether the decrease in cell growth was due to an increase in apoptosis, cells exposed to CSC were detached and the percentage of viable or apoptotic cells was determined by means of an Annexin V-PI kit by flow cytometry after 5 days of incubation. As shown in Figs. 2A and 2B, the viable cell percentage was significantly higher in the control cells (80.7 ± 4.9%) than in the CSC-exposed cells in a dose-dependent manner (41.7 ± 6.9% with 10% CSC, p = 0.01; 34.9 ± 7.2%

with 30% CSC, $p = 0.01$; and 25.4 ± 14.0% with 50% CSC, $p = 0.05$) (Fig. 2B). The number of apoptotic cells significantly increased with 10, 30, and 50% CSC compared to that observed in control cells. Similar results were obtained at 7 and 14 days (data not shown). CSC-induced cell growth decreases possibly took place through a deregulation of the cell cycle progression. To monitor the effect of CSC on the cell cycle, we treated gingival fibroblasts with various concentrations of CSC for 5 days, after which time we quantified the cell percentages in the different cell cycle phases. In the control group, less than 70% of the cells were in the G0/G1 phase, while over 10% were in the S phase and over 15% were in the G2/M phase (Fig. 3). However, following exposure to CSC, the values were significantly ($p < 0.05$) reduced. In contrast, in the fibroblast culture exposed to 10% CSC, over 80% of the cells were in the G0/G1 phase, with approximately 5% in S phase and close to 12% in the G2/M phase. Similar results were obtained with fibroblasts exposed to 30% and 50% CSC.

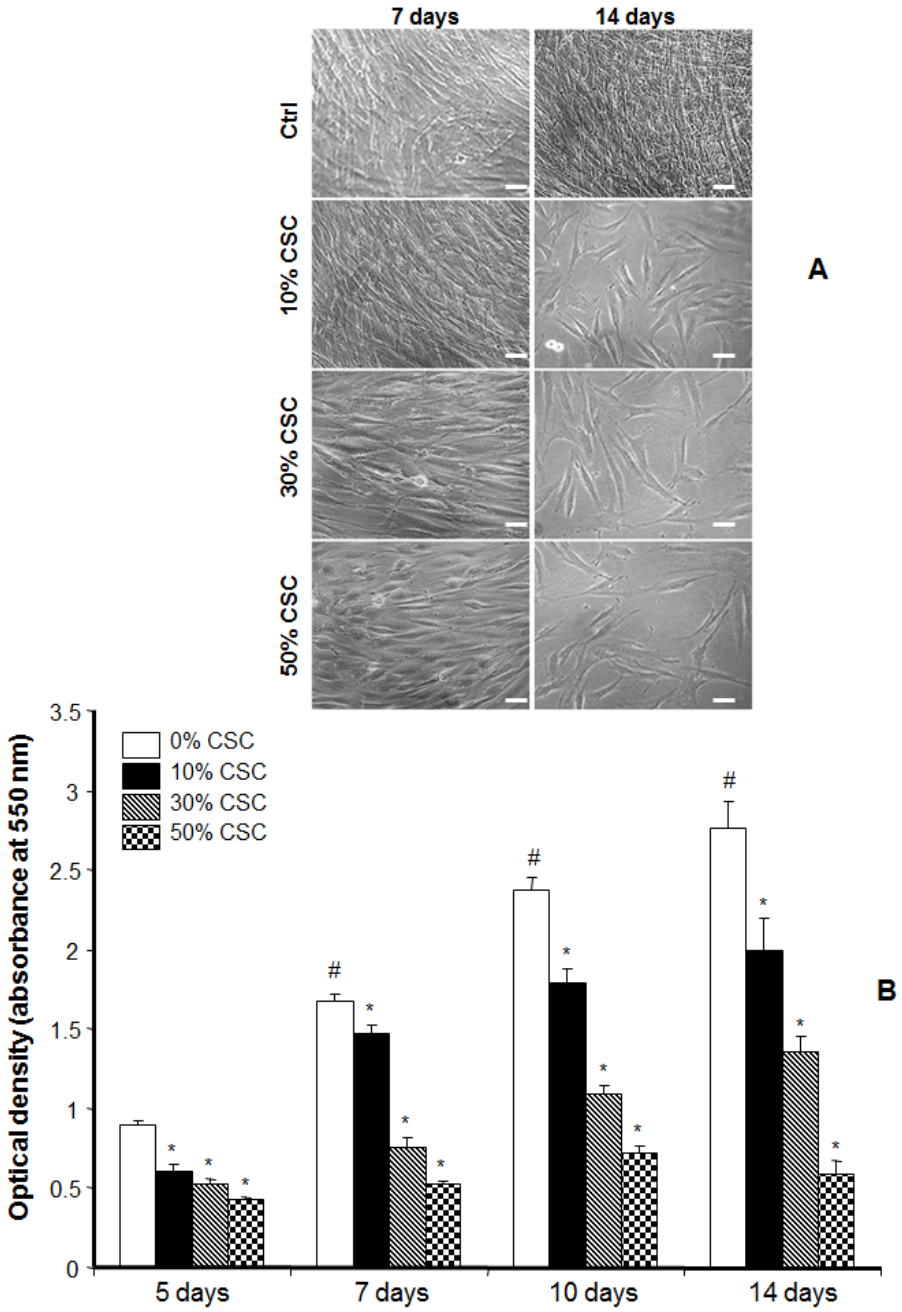

Figure 2-1: Effect of cigarette smoke condensate on gingival fibroblast viability. Gingival fibroblasts were seeded at 4 x 10^5 cells per well and incubated with or without CSC at various concentrations (0, 10, 30, and 50%) for 5, 7, 10, or 14 days. Photos were taken to determine cell shape (Panel A, Scale bars: 50 μm). MTT assay was performed to determine cell growth (Panel B). # = $p < 10^{-15}$ obtained by comparing cell growth at 5 days to 7, 10 and 14 days without exposure to CSC; * = $p < 10^{-10}$ compared to 0% CSC (control).

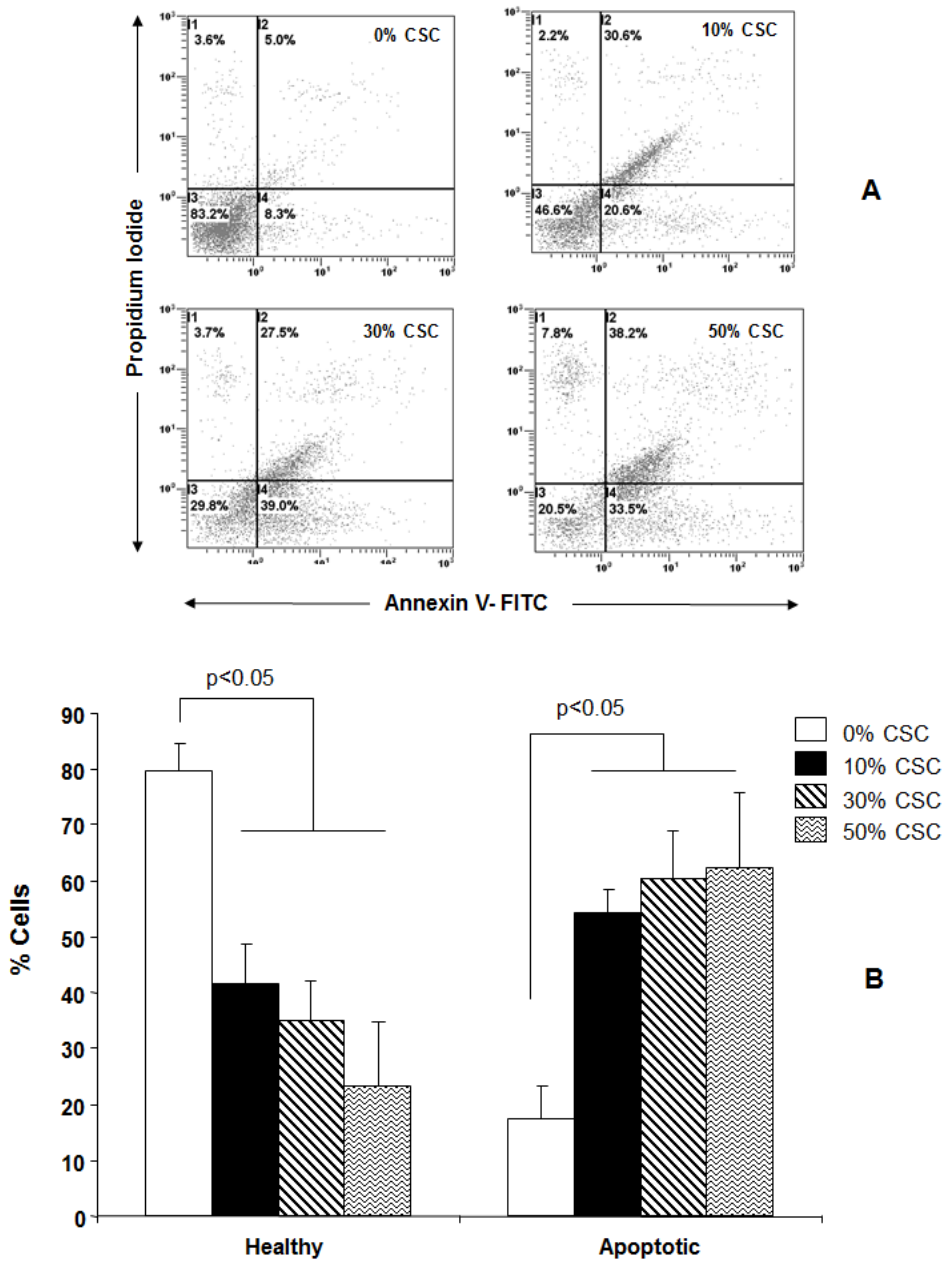

Figure 2-2: Gingival fibroblast survival after cigarette smoke condensate exposure. Gingival fibroblasts were seeded at 4 x 10^5 and stimulated with 0, 10, 30, or 50% CSC in DMEM/10% FBS for 5 days. Apoptosis was measured with an Annexin V-PI kit by flow cytometry. (A) Representative result of three different experiments. Quadrant I3: Viable cells, Annexin V- and PI-negative. Quadrant I4: Apoptotic cells, Annexin V-positive and PI-negative. Panel (B) refers to the percentage of viable and apoptotic cells compiled from three different experiments.

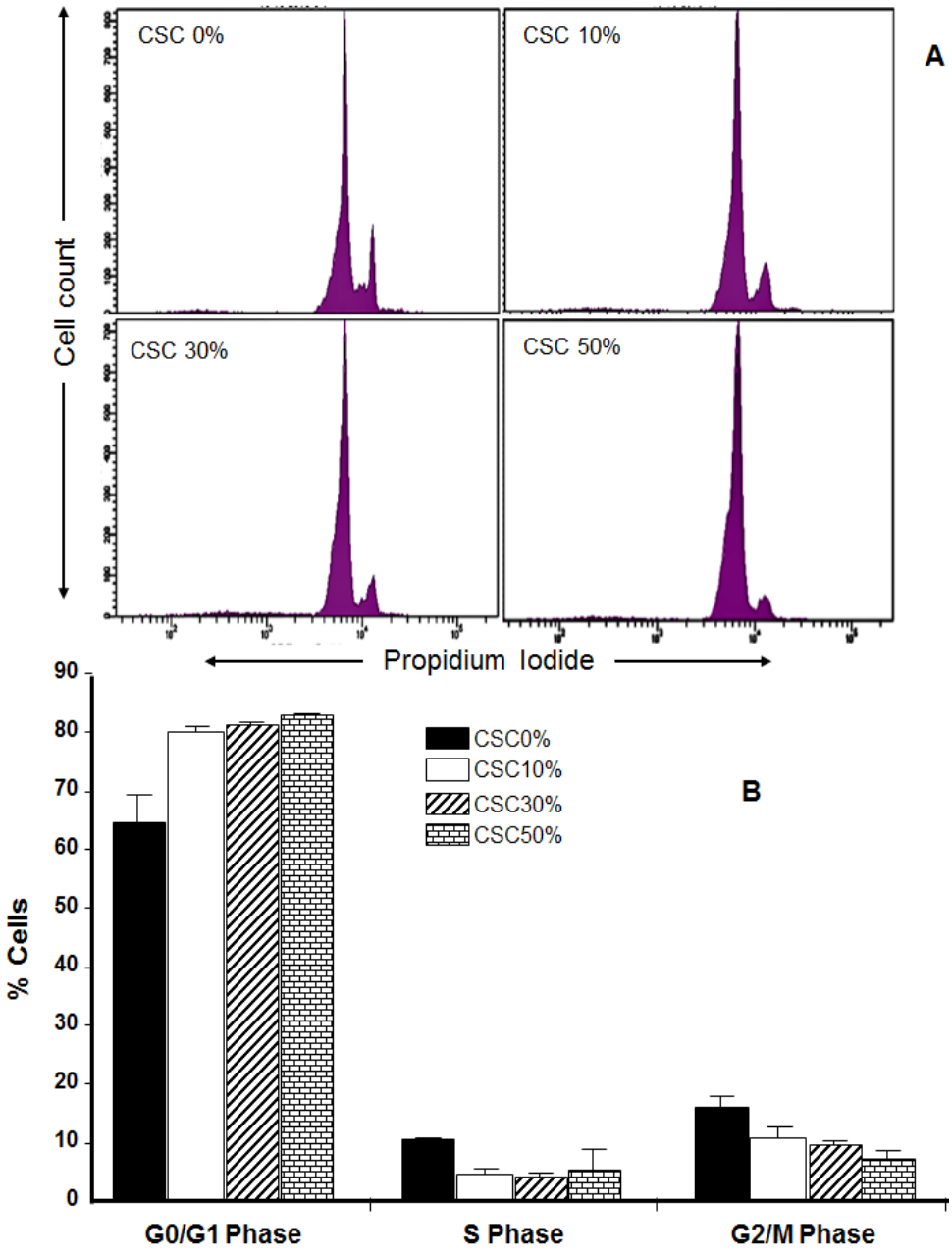

Figure 2-3: Effect of cigarette smoke condensate on cell cycle progression in gingival fibroblasts. The cell cycle phases were analysed following exposure of gingival fibroblasts to various concentrations (0, 10, 30, or 50%) for 5 days. The cells were then subjected to PI staining and cytometry analysis. Results are means ± SD (n = 3 for Fig. 3B).

2.4.3 Cigarette smoke condensate activated the apoptotic genes of human gingival fibroblasts. Because CSC increased the apoptotic activity of human fibroblasts, this possibly occurred due to the modulation of different apoptotic genes. To test this hypothesis, we performed real-time PCRs with arrays of 88 primers specific to genes involved in the apoptotic process following stimulation with 30% CSC during 5 days. Table 1 shows the genes displaying a significant fold change of gene expression after CSC exposure. TNF-induced apoptosis receptors 10A and 10B were strongly and significantly induced by the CSC. In addition, caspases 7 and 10, Bax and p53 were all overexpressed following cell exposure to the condensate. Among the repressed genes, Bcl-2-related protein A1 was greatly downregulated compared to what was observed in the fibroblasts not exposed to CSC (Table 2).

2.4.4 Cigarette smoke condensate induced Bax and p53 protein expression by human fibroblasts. As CSC caused the overexpression of several apoptotic genes in the human gingival fibroblasts, we examined whether this overexpression was mirrored with an increased protein production. We thus selected two key apoptotic proteins, Bax and p53, for this study. Using Western blot analysis on gingival fibroblasts after a 5-day exposure to 30% CSC, we revealed a significant induction of Bax and p53 proteins (Fig. 4). Indeed, both Bax and p53 proteins were significantly overproduced by almost 2-fold in the CSC-stimulated cells (Fig 4B). β-actin was used as a loading control and did not vary between the controls and the CSC-stimulated cells. Bax and p53 protein expression also increased when the cells were exposed to 10 or 50% CSC for 5 days (data not shown).

Table 2-1: PCR arrays showing apoptotic activated/repressed genes following exposure of human gingival fibroblasts to cigarette smoke condensate.

Gene	Description	Fold Change
TNFRSF10A	TNF receptor superfamily, member 10a	2.66
TNFRSF10B	TNF receptor superfamily, member 10b	2.01
PYCARD	PYD and CARD domain containing	1.95
BNIP3L	Bcl2 interacting protein 3-like	1.84
CASP8	Caspase 8	1.73
CD40	CD40 molecule	1.73
BCL10	B-cell lymphoma 10	1.64
CASP7	Caspase 7	1.59
CASP10	Caspase 10	1.58
DIABLO	DIABLO, IAP-binding mitochondrial protein	1.58
FAS	Fas (TNF receptor superfamily)	1.56
TP53	Tumor protein p53	1.55
TNFRSF1B	TNF receptor superfamily, member 1B	1.53
CASP6	Caspase 6	1.52
BAX	Bcl2-associated athanogene	1.52
BFAR	Bifunctional apoptosis regulator	1.48
BNIP3	Bcl2 interacting protein 3	1.48
LTBR	Lymphotoxin beta receptor	1.44
LTA	Lymphotoxin alpha	-1.45
BCLA1	Bcl-2-related protein A1	-1.63
BIRC3	Baculoviral IAP repeat containing 3	-1.71

2.4.5 Cigarette smoke condensate increased caspase-3 activity in the gingival fibroblasts. Caspases are essential to the apoptotic response. Because some caspase genes were overexpressed when the fibroblasts were exposed to CSC, we investigated caspase activity by measuring caspase-3 activity in gingival fibroblasts following stimulation with CSC for 5 days. We then compared the activity of the CSC-stimulated cells with that of the control cells to calculate a percentage of caspase-3 activity (Fig. 5). Cells grown in control medium for 5 days recorded 100 ± 28% caspase-3 activity, while cells grown with medium supplemented with 10, 30, or 50% CSC showed higher caspase-3 activity ranging between 180 ± 42% and 190 ± 50%, which was a significant increase ($p \leq 0.01$) compared to that recorded by the control. Caspase-3 activity was also measured after short (6 to 24 h) and long (7, 10, and 14 day) incubation times with CSC, with no significant increase of caspase-3 activity reported (data not shown).

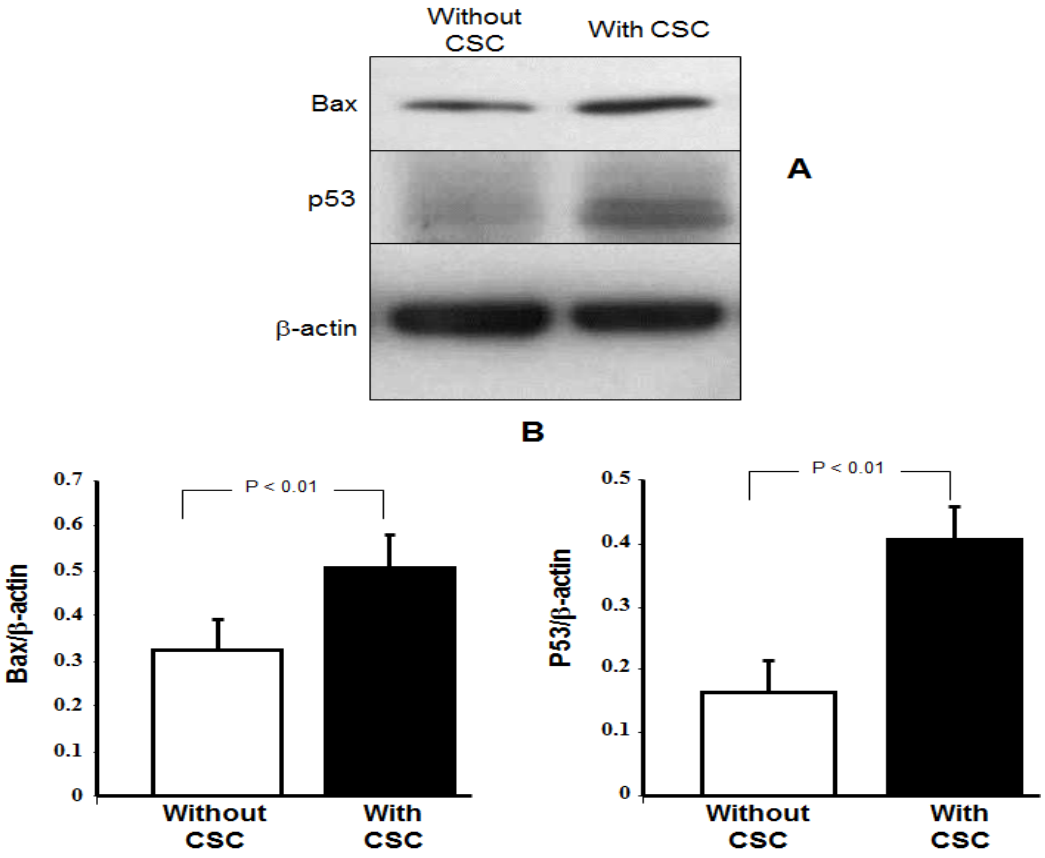

Figure 2-4: Modulation of Bax and p53 protein expression by cigarette smoke condensate. Gingival fibroblasts were exposed to 0 or 30% CSC for 5 days and the total protein extracts were prepared. Bax and p53 expression was determined by Western blotting, and then scanned using Genetools software (Panel B). A β-actin antibody was used as the loading control. The figure is representative of three different experiments.

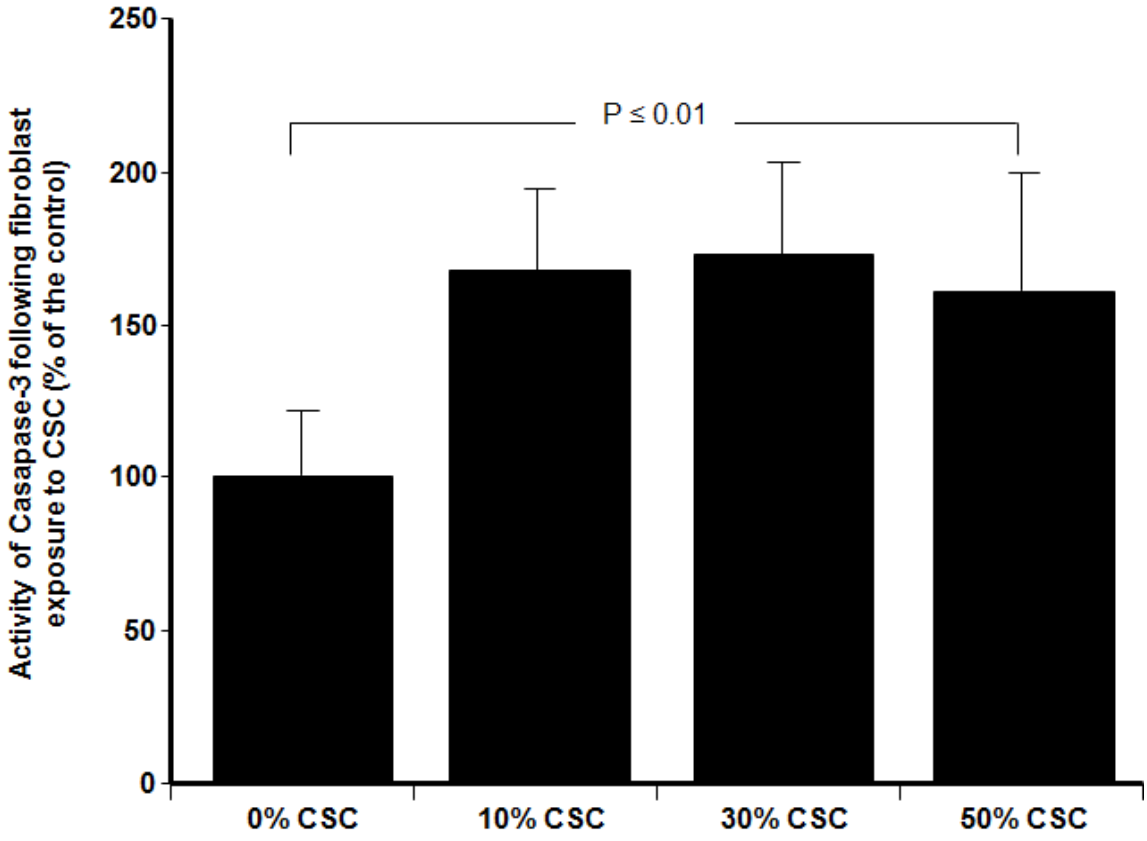

Figure 2-5: Caspase-3 activity following cigarette smoke condensate stimulation. Gingival fibroblasts were exposed to CSC for 5 days and then suspended in lysis buffer. Protein extracts (100 µg) were assayed for caspase-3 activity by means of a CPP32 activity kit. The optical densities of the control cells and CSC-exposed cells were compared to determine a percentage of CPP32 activity. Data shown are the means SD of the casapase-3 activity in the samples calculated as a percentage of the control (non-CSC-exposed cells in which the caspase-3 activity was considered 100%). (n = 3).

2.4.6 Bax and caspase-3 displayed cytosolic and mitochondrial co-localization. The subcellular location of Bax in the human gingival fibroblasts was determined by means of Zeiss Apotome fluorescence microscopy (Fig. 6) showing a diffuse cytosolic staining pattern with a superimposed punctate perinuclear component; low levels of diffuse nuclear staining were also observed (green

staining). The punctate component appeared similar to that observed with the mitochondria (red staining) following staining with mitochondrion-selective vital dye (MitoTracker, Life Technologies). Double staining with anti-bax and Mito-Tracker revealed that the punctate fraction of Bax co-localized with the mitochondria (overlapping red and green pixels seen as yellow). Similar analyses were conducted to localize caspase-3 in the CSC-exposed gingival fibroblasts, showing caspase-3 as co-localized with the mitochondria (Fig. 7). Overall data thus confirm that Bax and caspase-3 displayed cytosolic and mitochondrial distribution in the CSC-exposed gingival fibroblasts.

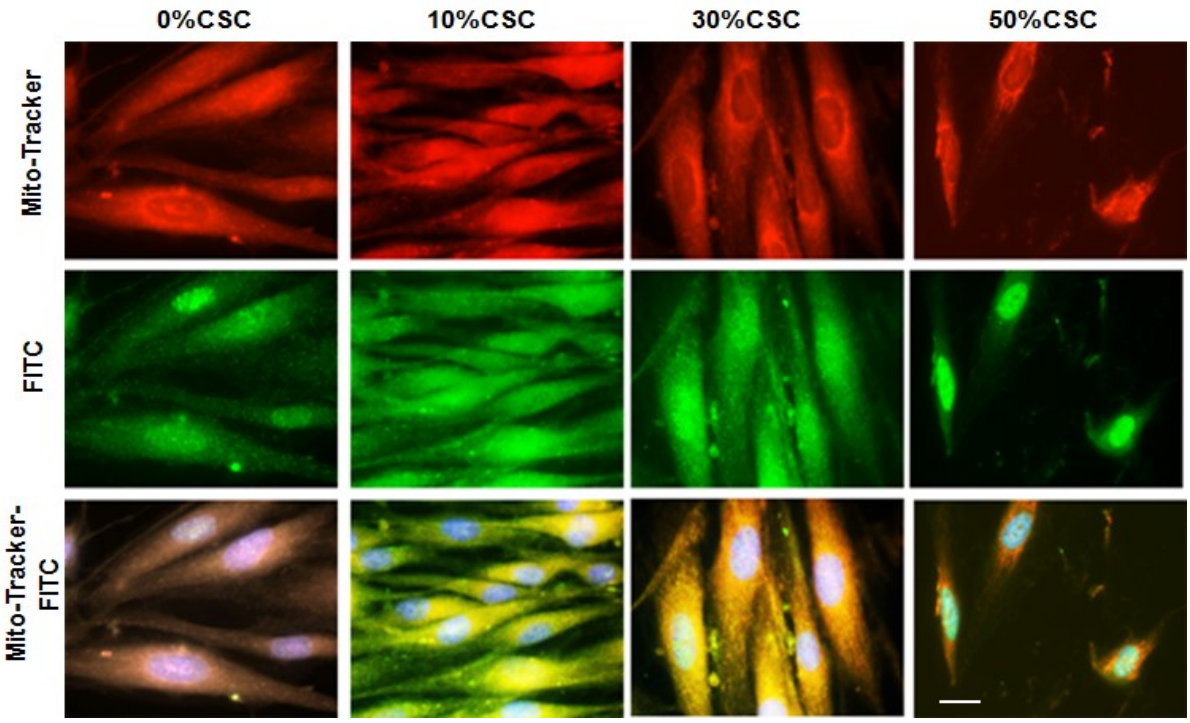

Figure 2-6: Bax localized in the cytosol and mitochondria of cigarette smoke condensate - exposed gingival fibroblasts. Following exposure to CSC for 5 days, cells were labeled with MitoTracker, fixed, permeabilized, and stained with mouse anti-human Bax monoclonal Alexa Fluor 488-conjugated secondary antibody. The stained cells were then examined under a Zeiss Apotome microscope. Bax-positive cells visualized with fluorescein were assigned the color green, while mitochondria labeled with MitoTracker were assigned the color red. When red and green images merge, the overlapping red and green pixels appear orange/yellow. Bar: 10 μm.

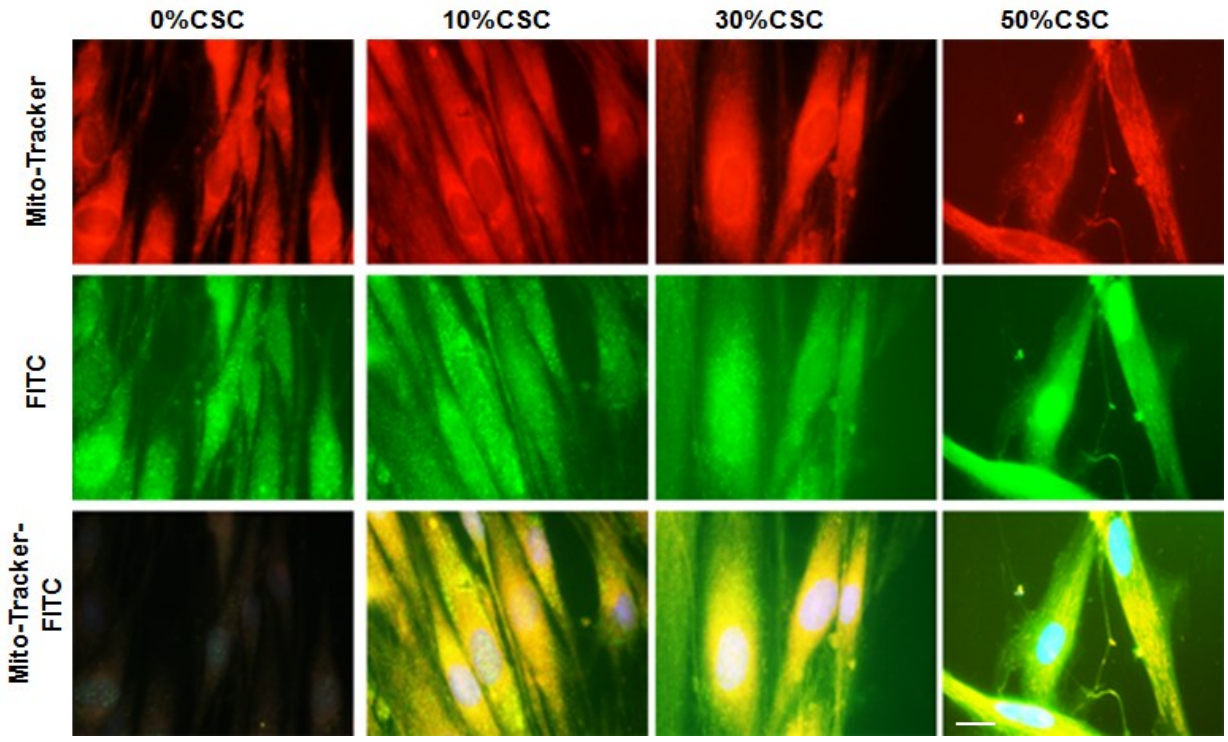

Figure 2-7: Caspase-3 localized in the cytosol and mitochondria of cigarette smoke condensate -exposed gingival fibroblasts. Following exposure to CSC for 5 days, gingival fibroblasts were labeled with MitoTracker, fixed, permeabilized, and stained with mouse anti-human casapse-3 monoclonal antibody, followed by Alexa Fluor 488-conjugated secondary antibody. The stained cells were then examined under a Zeiss Apotome microscope. Caspase-3-positive cells were visualized with fluorescein and assigned the color green, while mitochondria labeled with MitoTracker were assigned the color red. When red and green images merge, the overlapping red and green pixels appear orange/yellow. Bar: 10 μm.

2.4.7 Cigarette smoke condensate decreased IL-6 and IL-8 secretion by the gingival fibroblasts. As shown in Fig. 8A, fibroblasts not exposed to CSC produced a high basal level of IL-6 after 24 h of culture under the appropriate conditions, while the cells exposed to CSC produced low levels of IL-6. The effect of CSC on IL-6 production was observable with each tested concentration. Of interest is that the low levels of IL-6 secreted by the fibroblasts were obtained

with an elevated CSC concentration. The decrease of IL-6 following cell exposure to CSC remained observable at 48 and 72 h (Fig. 8A). IL-8 was also modulated by the CSC. As shown in Fig. 8B, the unexposed fibroblasts produced a basal level of IL-8, which was significantly higher than that produced by the CSC-exposed cells. The reduction was observed with each tested CSC concentration. The effect of CSC on IL-8 secretion thus appeared to be linked to concentration level. Similar results were obtained at 48 and 72 h (Fig. 8B). We noted that the greater the CSC concentration, the lower the level of secreted IL-8.

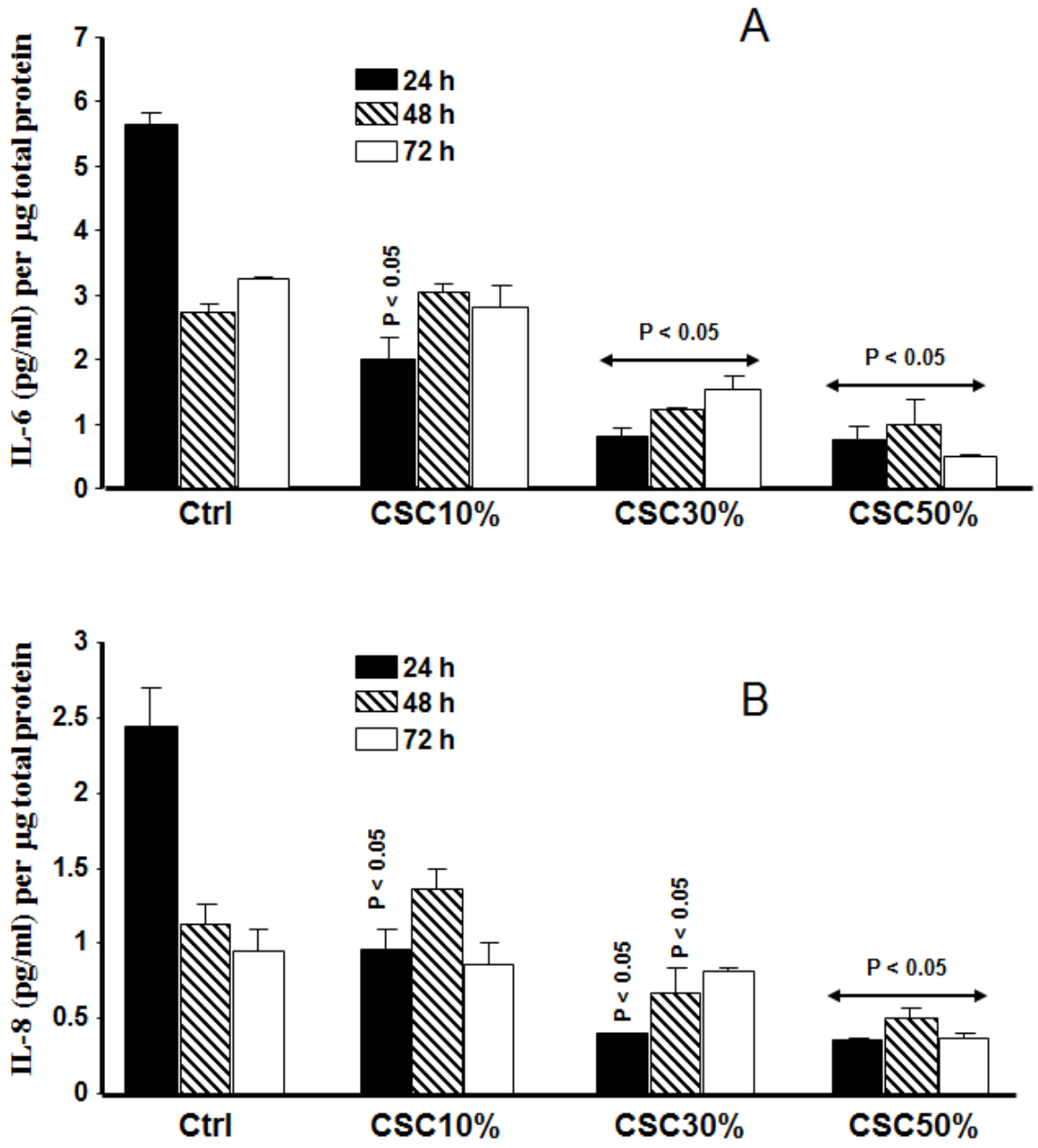

Figure 2-8: Cigarette smoke condensate inhibited IL-6 and IL-8 secretions by gingival fibroblasts. Fibroblast cultures were exposed to various concentrations of CSC for 24, 48, and 72 h. Supernatant was then collected from each condition and used to measure IL-6 (panel A) and IL-8 (panel B) levels by ELISA. Data are expressed as means ± SD.

2.5 Discussion

In the present study, we demonstrated that the continuous exposure of normal human gingival fibroblasts to CSC resulted in shape change and growth inhibition as confirmed by optical microscope observations and MTT. Cigarette smoke induced both apoptosis and necrosis as of human gingival fibroblasts. Cells exposed to CSC showed significant increase in apoptotic/necrotic cell density, as ascertained by annexin-V/propidium iodide staining. These results are consistent with those reported for the effects of CSC on human bronchial (22) and nasal epithelial cells (23). The decreased growth of gingival fibroblasts following exposure to CSC may have occurred due to cell-cycle deregulation (24), as demonstrated in this study, where CSC increased the G0/G1 phases and decreased the S and G2/M phases, compared to that observed with the non-exposed cells. Our results also support a previous study showing that cigarette smoke compounds such as nicotine inhibited cell cycle progression by inducing the G_1 arrest of various cell lines including HaCaT, IHOK, HN4, and HN12 (25). Taken together, the cell damaging effects of CSC on gingival fibroblasts might potentiate inflammation, leading to periodontal disease.

Our PCR array data demonstrate that CSC modulated gene expression in gingival fibroblasts involved in the apoptotic/necrotic pathways, including Bax, previously shown to relate to apoptosis (26). Bax upregulation may be involved in the modulation of fibroblast apoptosis through the disruption of the pro-apoptotic–anti-apoptotic balance in the mitochondrial apoptosis pathway, as previously reported (27). Furthermore, by the opening of permeability transition pores, the activation and translocation of Bax to the mitochondria may induce the release of apoptotis-inducing proteins into the cytosol (27).

Apoptosis onset is associated with the proteolytic activation of caspases (28). Caspases are synthesized as proenzymes that are processed by self-proteolysis and/or cleavage by another protease to their active form in cells undergoing apoptosis (29). Caspase-3, a major apoptosis trigger, is increased during early apoptosis, and the activated form is a marker for cells undergoing apoptosis (30). In this study, the gingival fibroblasts exhibited significant caspase-3 activity and Bax expression during the long exposure time to CSC. The induction of Bax and caspase-3 suggest that CSC induced these two signals, thus promoting fibroblast apoptosis and suggesting that CSC used a mitochondria-related signal pathway to induce fibroblast apoptosis by inducing translocation of Bax and casapse-3 to the mitochondria. This translocation has been shown to alter the outer mitochondrial membrane permeability and subsequently lead to the release of pro-apoptotic proteins, such as cyto *c* and AIF (31).

In promoting gingival fibroblast apoptosis, CSC may also hamper cell growth through specific genes, such as *p53.* This gene is one of the most important intracellular sensors to signal stress and damage (e.g., DNA damage) (32). P53 is capable of initiating apoptosis by signaling toward mitochondria, with the subsequent induction of mitochondrial dysfunction translating to a loss of membrane potential and mitochondrial outer membrane permeabilization through the activation of pro-apoptotic proteins (32). These findings support our study showing that CSC increased *p35* gene expression and protein production by gingival fibroblasts. These results are also in agreement with those of previous studies reporting an increase of P53 in cigarette smoke-exposed cells, including endothelial and epithelial cells (33,34). Future studies using normal human gingival cells integrated into an engineered connective tissue will enable us to learn more regarding the cellular and molecular pathways involved in the interactions between cigarette smoke and human tissues and ultimately, periodontal disease development.

Periodontitis occurs as a result of bacterial infection, tissue damage and inflammation. Although it is unclear which comes first, our studies suggest that cigarette smoke and cell damage could enhance this response. Exposure to cigarette smoke of cultured human cells including fibroblasts has been reported to stimulate the release of different mediators including IL-8, GM-CSF, IL-1β and TNF-α (35). However in other conditions, cigarette smoke inhibits proliferation of normal fibroblasts (36), induces cellular senescence (37) and inhibits alveolar repair (38). This is supported by our study showing that the secretion of IL-6 and IL-8 by gingival fibroblasts was downregulated by CSC, however, this is may not be a consequence of cell death. IL-6 is a proinflammatory cytokine produced by a number of cell types, including fibroblasts. The expression of IL-6 is lower in smokers than in non-smokers when using bronchoalveolar lavages (39) or alveolar macrophages (40). IL-6 secretion significantly decreased in bronchoalveolar lavage fluid of asymptomatic smokers compared to non-smoking subjects (41). Our findings are thus in agreement with these observations. However, in other studies, cigarette smoke increased proinflammatory cytokines including IL-6 in the lungs of mice (42) but also in gingival epithelial cells (43). The contrasting effects (increase or decrease) of cigarette smoke on proinflammatory cytokines may be explained by the differences with regard to experimental model and exposure regimes, among others.

Interleukin-8 is another key inflammatory mediator regulating neutrophil infiltration, resulting in inflammation in various tissues (44). This cytokine may be deregulated by such agents as cigarette smoke. In this study, we showed that CSC reduced the secretion of IL-8 by gingival fibroblasts. These data are in agreement with those reported in the literature (45). Acrolein (AC), a major component of cigarette smoke decreased IL-8 gene expression and protein secretion by primary human sinonasal epithelial cells (45). Furthermore, cigarette smoke reportedly decreased IL-8 production by epithelial cells in COPD patients (45). Thus the decrease in IL-8 release in the CSC-

exposed gingival fibroblasts may not be explained by cell death, but possibly by the decrease of their activities following contact with CSC. Further studies should focus on the mechanisms of CSC contributing to the decreases of IL-6 and IL-8.

In summary, we demonstrated that exposure to CSC significantly reduced normal human gingival fibroblast growth. This occurred through an apoptosis pathway involving Bax, caspase-3, and p53. CSC was also able to suppress the inflammatory and innate immune function of gingival fibroblasts by decreasing IL-6 and IL-8 secretion, likely related to the decrease of cell growth in conjunction with high apoptosis following exposure to CSC. The effects of CSC on gingival fibroblasts can compromise the interaction between gingival epithelial cells and fibroblasts, resulting in periodontal disease development and potentiating inflammation due to tissue damage. Additional research on the mechanism of action of cigarette smoke will be necessary to provide insight regarding the connection between cigarette smoke, fibroblast dysfunction, and oral inflammatory diseases such as periodontitis.

2.6 Conflict of interests

The authors declare that they do not have any conflict of interests.

2.7 Acknowledgments

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Chapter 3

Article 2

Repeated exposure to cigarette smoke condensate induced gingival fibroblast proliferation through the activation of cell cycle genes and telomerase activity.

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Running title: Effect of cigarette smoke condensate on gingival fibroblasts

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Foreword

This article includes an introduction, an experimental protocol, results, discussion and conclusion. This publication was obtained thanks to the contribution of the following authors: **Alamri A**, Jacques E, Semlali A, Rouabhia M.

Alamri A conducted all the experiments with the help of Semlali A, Jacques E and Rouabhia M.

Alamri A, Semlali A, Jacques E and Rouabhia M, analyzed and interpreted the data.

Alamri A, Semlali A, Jacques E drafted the first version of the manuscript.

Mahmoud Rouabhia completed the manuscript.

All authors read and approved the published manuscript.

3.1 Abstract:

The objective of this study was to investigate the effect of repeated exposure to low concentrations of the cigarette smoke condensate (CSC) on the survival, oncogenes profiling, telomere and telomerase activity of human gingival fibroblasts. **Methods**: Primary human gingival fibroblasts were exposed three times a day, during 15 min to CSC at one of the following concentrations: 0, 2, 5 or 10%. The exposure periods were 10, 20 or 30 days. Cell proliferation was evaluated by mean of BrdU assay. Oncogenes & Tumor Suppressor Genes profiling was performed by using PCR Array. Protein telomerase detections were determined by protein telomerase assay. Telomere restriction fragment analysis was performed using the TeloTAGGG Telomere Length Assay Kit. **Results**: Exposure to low concentration of CSC led to a significant increase in cell growth. Cell proliferation was significant starting from 10 days post exposure and maintained up to 20 days. Gene profiling demonstrated that CSC modulates the oncogenes pathway increasing *CDK4, SRC JUND, NFKBIA* and *PIK/CA*, but decreasing other genes such as *CASP8, RARA*. Tumor suppressor genes pathway mostly showed repressed genes such as *BRCA2, WWOX* and *BRCA1*. When analyzing apoptosis genes pathway, multiple genes were repressed including *BCL2, MCL1*. This was supported by the repression of certain genes involved in cell cycling were we found decreased expression of *TP53, CCND1* and *BRCA2*. The changes in gene expressions were supported by telomerase activity analyses showing significant (p 0.01) increase when the cells were exposed to CSC for 10 and 20 days. It is interesting to note that, greater the CSC concentration higher was the telomerase activity. This is supporting the telomere length shortening following cell exposure to 10% CSC for ten days. **Conclusion**: CSC increased fibroblast cell division through the modulation of different oncogenic genes. CSC also increases telomerase activity and a slight telomere length shortening. This study suggests a role for chronic cigarette smoke exposure in the selection of

proliferative fibroblasts with potential cell cycle control gene deregulation that may lead to initiation cigarette smoke oral pathologies.

Keywords: Gene profiling; cigarette smoke; fibroblasts; PCR arrays, oncogenes, telomere, telomerase.

3.2 Introduction:

Cigarette smoke is a toxicant rich product reaching the tissues and the cells leading to multiple severe pathologies (1). Following burning of a cigarette two phases will be generated known as particulate and gaseous phases. The particulate phase contains polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, and metals (2). These are most frequently associated with cancer (3). The particulate phase is also rich in long-lived radicals, including semiquinone, which forms hydroxyl radicals and hydrogen peroxide when it reacts with the superoxide anion (2,3). The gaseous phase contains aldehydes (formaldehyde, acrolein, and acetaldehyde). They are associated with chronic pulmonary disease and the lung toxicology (2,3). Also, this gaseous phase contains short-lived oxidants, such as superoxide anion and nitric acid leading to the formation of a highly reactive peroxynitrite (4). Gaseous and particulate CS constituents first interface with the immune system at the mucosal surfaces lining the oral cavity, sinuses, and airways (5). These damage the cells leading to lipid peroxidation, activation oxidative pathways, and DNA damaging (6).

Tobacco products are causally linked to a variety of cancers, including those of the lung, oral cavity, nasal cavity, larynx, oropharynx, hypopharynx, oesophagus, stomach, liver, pancreas, bladder, ureter, kidney, cervix, and myeloid leukaemia (7,8). There is a dose-dependent association between tobacco smoking and oral cancer. Also, the risk for cancer development is associated with the number of daily smoked cigarettes and the duration of smoking (9, 10). Deregulation of cell proliferative pathways is central to cancer initiation and progression (11); however, the effect of CS on cell proliferative pathways in cancer cells still needs to be investigated. Some available studies using 10-day exposure of lung cancer cells to total tobacco condensate reported an increased tumorigenicity of the exposed cells when grafted into in nude mice (12). Furthermore, it was observed that chronic cigarette smoke exposure inhibited TGF-β signaling but increased cell tumorigenic phenotype (13). Conversely, other studies showed that CSE induced epithelial cell line

senescence through cell growth arrest, an increased senescence-associated β-galactosidase activity and p21 (14). These data are interesting, but not reflecting what may happen with normal human cells such as gingival fibroblasts when exposed to cigarette smoke.

Gingival fibroblasts are elongated cells of mesodermal origin, with a fusiform or spindle-like shape (15). They are the most abundant cells populating the extracellular matrix (ECM) forming the connective tissue. Through this ECM, fibroblasts are tightly interacting with epithelial cells ensuring gingival mucosa tissue homeostasis (16). Fibroblasts are actively involved in regulating tissue remodeling and repair. Immediately after damage, healthy fibroblasts proliferate and acquire different healing properties such as the expression of α–smooth muscle actin, fibronectin, taking a leading role in the wound healing process (17, 18). Furthermore, fibroblasts interact with epithelial cells to maintain their homeostasis and to prevent the growth of neoplastic cells (19,20). Exposure of gingival tissue to cigarette smoke may deregulate epithelial structure (21) reaching the connective tissue, thus deregulating gingival fibroblasts properties. The goal of this study was to evaluate the effect of repeated exposures to cigarette smoke condensate on primary human gingival fibroblast viability, proliferation and telomere/telomerase expression. We also analysed the effect of CSC on oncogene genes' expression.

3.3 Materials and Methods

3.3.1 Preparation of the cigarette smoke condensate: To prepare cigarette smoke condensate, we used 1R3F cigarettes purchased from the Kentucky Tobacco Research & Development Center (Orlando, FL, USA). Each cigarette was placed into one end of a silicone tube linked to an Erlenmeyer flask containing 100 mL of 0.09% sodium chloride. On the other end, a second silicone tube linked to the Erlenmeyer was connected to a standard vacuum. The cigarette was attached to the cigarette holder, lit, and the smoke was extracted by applying vacuum, pulling the smoke directly into the 0.09% sodium chloride solution. The procedure was repeated for a total of five whole cigarettes. The resulting cigarette smoke condensate (CSC) solution was then sterilized by filtration through a 0.22-μm filter, aliquated and frozen at -20°C until use.

3.3.2 Gingival fibroblast cell culture: Small samples of the oral mucosa were collected from gingival graft patients following their informed consent. This protocol was approved by the Laval University Ethics Committee. Biopsies were collected in conjunction with surgical procedures. The tissues were treated with thermolysin (500 μg/mL) to separate the epithelium from the lamina propria (22). To isolate the gingival fibroblasts following separation from the epithelium, the lamina propria was placed in a collagenase P solution (0.125 U/mL; Boehringer Mannheim, Laval, QC, Canada) for 45 min at 37 °C to extract the fibroblasts. Isolated cells (2×10^6) were seeded in 75-cm^2 flasks (Falcon, Becton–Dickinson, Cockeysville, MD, USA) and grown in Dulbecco's modified Eagle (DME) medium (Invitrogen) containing 10% fetal calf serum (Invitrogen). The fibroblasts were used once the cultures reached 90% confluence.

3.3.3 Effect of repeated exposure to cigarette smoke condensate on gingival fibroblast proliferation: Primary gingival fibroblasts were seeded in 6 well plates at 10^4 cells per well in 10% FBS-supplemented DMEH medium and were incubated in a humidified atmosphere containing 5%

CO_2 at 37°C. When the culture reached approximately 50% confluence, the fibroblast cell culture was subjected to CSC stimulation. The tested concentrations of CSC were 0% 2%, 5% or 10%. The exposure time was three times a day during 15 min each time. After each exposure period, cultures were washed twice with warm medium then 10% FBS-supplemented medium was added to the culture followed by incubation at 37C in a five% CO2 humid atmosphere. This CSC-repeated exposure regime was performed for 10, 20 or 30 days. On the last day of each exposure period, cell cultures were pulsed with BrdU to evaluate the cell proliferation using BrdU colirimetric kit (Roche, Basel, Switzerland)**.** Briefly, the cells were incubated with BrdU for four h; then cell DNA was denatured in one step using FixDenat. The anti-BrdU-POD was bound to the BrdU that had been incorporated into newly synthesized cellular DNA. The immune complexes were detected through the subsequent substrate reaction. Before the reading, 25 µl of 1 M H_2SO_4 was added to each culture and was incubated for 1 min on a shaker at 300 pm to stop the reaction. The reaction product was quantified by measuring the absorbance at the respective wavelength 370 nm with a reference wavelength of 492 nm using ELISA reader (BioRad).

3.3.4 Human oncogenes and tumor suppressor gene profiling through quantitative real-time PCR array: Following repeated exposure to CSC, fibroblasts were used to extract total RNA by means of an RNAspin mini kit (GE, Mississauga, ON, Canada) according to the manufacturer's instructions. RNA concentration and quality were determined with the Experion automated electrophoresis station (BioRad, Mississauga, ON, Canada). RNA quality was considered optimal in any sample with 28S/18S rRNA ratios above 1.8. Real-Time PCR arrays were performed using the RT^2 Profiler PCR (PAHS-502Z) Array System from SuperArray Bioscience (Frederick, MD) following the manufacturer's instructions. Table-1 shows the different genes included that will be investigated. Briefly, total RNA (1000 ng) was used to prepare cDNA with the RT^2 first strand kit

(QIAGEN, Germantown, MD, USA). PCR arrays containing 88 gene-specific primers related to oncogenes were amplified. Results were analyzed using the $^{\Delta\Delta}$Ct method and fold changes between 0% and 10% CSC stimulated samples were calculated. Genes were selected if a fold change above 1.3 was observed and if baseline cycle thresholds for this gene were below 30 to eliminate feebly expressed genes.

3.3.5 Telomerase activity assay: Telomerase activity was measured using the TRAPeze telomerase detection kit (Chemicon International, XBillerica, MA, USA) following the manufacturer's instructions. Briefly, fibroblasts were seeded into six-well plates at 10^4 cells/well, and then cultured for 24 h before exposure to CSC. Cells were exposed or not to CSC 3x15 min each culture day for 10, 20 or 30 days. Cells were then detached from the culture flasks, pelleted by centrifugation, then re-suspended in CHAPS lysis buffer. Protein levels were determined by bradford assay. Protein extracts (1.5 ug) were used in a Telomeric Repeat Amplification Protocol (TRAP) using primer mixes and iTaq polymerase (BioRad) to amplify telomeric repeats. Amplification was performed in a MyCycler (Biorad) with an incubation at 30ºC for 30 minutes followed by 33 cycles of 30 seconds at 94ºC, 30 seconds at 59ºC and 1 minute at 72ºC. PCR products were migrated on a 0.5X TBE, 10% acrylamide gel and visualized using a Chemicon Bioimaging system (Chemicon International) after staining with 1ug/ml ethidium bromide for 30 minutes. Data was analyzed using Genetools software (Syngene, Frederick, MD, USA). The relative telomerase activity of each extract was determined by taking the ratio of the entire telomerase ladder to that of the internal control. Experiments were controlled with a primer-dimer/PCR negative control and a telomerase-positive control included in the kit.

3.3.6 Telomere length: Primary human gingival fibroblasts were seeded into six-well plate at10^4 cells/well, and then cultured for 24 h before exposure to CSC. Cells were exposed or not to CSC

3x15 min each culture day for 10, 20, or 30 days. Cells were then used to extract total DNA. The average telomere length was measured using the Telo-TAGGG Telomere Length assay (Roche, Mississauga, ON, CA) following the manufacturer's instructions. Total DNA was extracted from cultured fibroblasts using the DNA Isolation kit for cells and tissues (Roche). DNA (1ug) was digested using a Hinf I / Rsa I restriction enzyme mixture at 37ºC for 2 hours and migrated on a 1X TAE / 0.8% agarose gel. Southern transfer of digested DNA on nitrocellulose membranes was performed using 20X SSC buffer overnight by capillary transfer. Membranes were crosslinked with UV light, blocked, washed and incubated with Anti-DIG-AP antibodies for 30 minutes. Detection was performed with chemiluminescence reaction and visualized on X-ray films and using a Chemicon Bioimaging system. Data was analyzed using Genetools software (Syngene). The mean telomerase length was calculated as a ratio of the intensity of the smear by the mean size of the smear compared to the molecular weight marker.

3.4 Results

3.4.1 Repeated exposure to cigarette smoke condensate promoted fibroblast proliferation: Primary human gingival fibroblasts cells were treated with CSC, and cell proliferation was evaluated by BrdU assay. As shown in Fig. 1, cells exposed three times a day for ten days showed significant ($p < 0.04$) proliferation as compared to non-exposed cells. Results are comparable whether the cells were exposed to low (2%) or high (5 a% or 10%) levels of CSC. After 20 days post exposure to CSC, we still have a significant ($p < 0.01$) cell proliferation following repeated exposure to CSC at 5 and 10%. However, after 30 days, there was no significant effect on cell proliferation. Both CSC exposed and non-exposed cell show comparable proliferation rates. Overall all these data demonstrated the repeated exposure to CSC lead to fibroblast proliferation at 10 and 20 days but not at late (30 days) exposure periods. Fibroblast proliferation under related exposure to CSC may involve different genes that control cell cycling cell apoptosis, etc.

3.4.2 Repeated exposure to cigarette smoke condensate modulates genes that promote oncogenesis: The gene profiling results revealed that multiple genes were either up or down-regulated following CSC. These genes are involved in all phases of cell cycling and are implicated in many signaling pathways crucial to the proliferation process, such as oncogenes, tumor suppressor genes, transcription factors, apoptosis, cell adhesion molecules and cell cycle. Only those with fold changes ≥ 1.3 or ≤ 0.8 were taken into account in our analyses.

Among the 12 genes involved in oncogene pathway, only *SRC* and *CDK4* gene were found upregulated at 10 days post exposure to CSC (Table 2). However, three genes *CASP8, REL* and *JUND* were found down-regulated all other involved in oncogene pathway were not modulated. At 20 day post exposure to CSC we found *JUND, NFKBIA* and *PIK3CA* were up-regulated (Table 2).

But *SRC, RARA, NRAS, RAF1, ETS1, AND MCL1* genes were down-regulated. Tumor suppressor gene pathway was also modulated. Table 2 shows that among the 7 genes we analysed only *MGMT* gene was up-regulated after 20 days post exposure to CSC. However, three genes, *BRCA2, TSC1* and *WWOX* were down-regulated in cells exposed 10 days to CSC. At 20 days, different genes *WWOX*, *BRCA1*, *HIC1* and *CKDN2B* were down-regulated.

CSC also modulates both oncogenes and tumor suppressor genes. As shown in Table 3, at 10 days post exposure there was only one gene ESR1 activated, but none at 20 days. However, three were down-regulated at 10 days; these are *MYCN, HRAS* and *BDR* genes. At 20 days, we found that *BCR, ESR1, TGFBA* and *KRAS* genes were down-regulated (Table 3). Genes involved in the transcription factors pathway were modulated by CSC. Table 3 showed that three genes PML, *JUN* and *RB1* were down-regulated after 10 days exposure to CSC. However, five genes *JUN*, *RB1, RARA, MYC* and *NF1* were decreased after 20 days post exposure to CSC. One common repressed gene at both 10 and 20 days of exposure to CSC was *JUN* gene.

Genes involved in apoptosis, cell adhesion molecule and cell cycle pathways were also modulated by CSC. Reported in Table 4, our data demonstrated that at 10 days two genes *BCL2* and *CASP8* were down-regulated. But, at 20 days there was 4 genes *BCL2, BRCA1, MCL1* and *MGMT* were down-regulated. Genes involved in cell adhesion molecules were modulated better at 20 days as compared to 10 days post exposure to CSC. One gene APC was increased at 10 days, however, the two other were unchanged. At 20 days, we found one activated gene which is APC, but two repressed genes which are NF1 and TGFB1.

Genes involved in cell cycle pathway were more modulated at 20 days compared to 10 days. Table 4 shows that one gene *BRCA2* was down-regulated, and one gene *CDK4* was up-regulated at 10

days post exposure to CSC. However, four genes *CCND1, BRCA1, TP53* and *CDKN2B* were down-regulated at 20 days post exposure to CSC.

Altogether oncogenes and tumor suppressor genes profiling demonstrated that CSC activated or suppressed different genes at 10 days as compared to 20 days. These effects may be linked to telomerase activity modulation leading to the selection of proliferative fibroblasts.

3.4.3 Cigarette smoke condensate increased telomerase activity in primary human gingival fibroblasts: Since there was increase of fibroblast proliferation and the modulation of different gene involved in oncogenes and tumor suppressor genes it is interesting to investigate the telomerase activity. As shown in figure 2, we found that CSC increased the telomerase activity of primary human gingival fibroblasts. Significant (p 0.01) increase was obtained with all tested CSC levels (2%, 5% and 10%). Also, a significant difference was obtained when comparing the telomerase activity at 2% to 5% and 10% of CSC. After 20 days post exposure, we still have high telomerase activity with CSC exposed fibroblast as compared to non-exposed cells. Also both 5 and 10% CSC levels were significantly increasing the telomerase activity as compared to the 2% CSC level. CSC exposed, and non-exposed gingival fibroblasts were subjected to telomere length evaluations. As shown in Fig 3, exposure to 2% CSC for 10 days did not change telomere length as compared to the control. However the exposure to 5% CSC led to a reduced size of telomere as shown by the dark DNA smear reaching almost 3 bp molecular weight. Exposure to 10% CSC showed important shortening of the telomere length; because the DNA smear reaches 1.5 bp. Comparable results were obtained at 20 days (data not shown). Shorter telomere may confirm the high cell proliferation we observed with BrdU staining. Thus, CSC exposed cells may escape harm effect of CSC by increasing telomerase activity preventing the telomere shortening.

3.5 Discussion

Smoking is a potent risk factor for periodontal diseases (24). Cigarette smoking impairs local immune defense to periodontal pathogen (25). Cigarette smoke effects can be by deregulating epithelial cell and or fibroblast behavior. In the present study, we looked at the effect of repeated exposure of primary gingival fibroblast to low and mid-levels of CSC. We were interested in the cell division process. Surprisingly we found that CSC exposed fibroblasts adopted active cell division process after 10 and 20 days repeated exposure. The increased cell division we reported in this study was supported by those demonstrating that cigarette smoke increased cell proliferation and deposition of collagen I, collagen III, and fibronectin of airway smooth muscle cells (26). Furthermore repeated exposure to low doses of cigarette smoke extract promoted normal bronchial epithelial cell proliferation (27). The cell proliferation we are demonstrating in this study may be due to the development of a sub-cell population that escaped the harm effect of CS chemicals and initiated proliferation process. Such hypothesis may be supported by previously reported study showing a subset of β-gal-negative population proliferating even after exposure to cigarette smoke extract (28). As suggested with lung fibroblasts (28) CSC might lead to two different gingival fibroblast phenotypes that include a non-senescent proliferative subpopulation. The fibroblasts that resist CSC-induced cellular senescence may contribute to the cigarette smoke-induced oral pathogenesis. Further studies are mandatory to support this hypothesis. Since CSC-exposed fibroblasts showed proliferation, we investigated the effect of CSC on different oncogene's pathways. Our study demonstrated that gingival fibroblast exposed to CSC express high level of *SRC* gene. This gene is known to play a role in the regulation of embryonic development and cell growth (29, 30). CSC promoting fibroblast proliferation may be through the activation of *SRC/V-SRC*. Indeed, it has been reported that through *v-Src* expression, some cell types such as neuroretina

cells become transformed and acquire sustained proliferative capacity (31). *V-SRC* is also involved in repressing cell differentiation (32,33), *V-SRC* mutants confirmed that this gene is implicated in the transformation induced alterations (34). Thus, our data suggest that CSC altered fibroblast proliferation process through *SRC* gene activation. CSC induced cell proliferation is supported by an increased expression of *CKD4* gene. This gene codes important proteins for cell cycle G1 phase progression through the D-CDK4/CDK6 protein complex (35). Thus, CSC may promote fibroblast cell cycle through G1 phase progression that involves *CKD4* gene. Cell proliferation needs nuclear gene activation such as *NFKBIA* (36). This gene encodes nuclear factor of kappa-B (*NF-kB*), a key transcription factor that moves between the cytoplasm and the nucleus leading to the activation of multiple genes including inflammatory genes (36). This would consolidate the fibroblast cell proliferation presented in this work but also confirms those previously reported data showing increased expression of cytokine by CS stimulated fibroblasts (37). This effect can be through the activation of certain cell oncogenes as we showed with exposed fibroblast expressing more PIK3CA. This oncogene was reported to be altered in several cancers (38). Consequently, we may hypothesis that CSC promotes fibroblast proliferation by activating *PIK3CA* oncogene. Further studies are needed to confirm or not this hypothesis. Cell proliferation can also be through the inhibition of certain apoptotic genes involved in controlling the cell cycle. Indeed, we found that CSC exposure leads the repression of *WWOX* gene expression in gingival fibroblast. *WWOX* gene encodes proteins promoting apoptosis (39). Repression of *WWOX* gene leads to cell cycle deregulation promoting cancer development (39, 40). The down-regulation of *WWOX* gene expression by CSC exposed fibroblast confirmed the proliferative non-apoptotic phenotype of our cells but not direct initiation of cell mutation to cancer cells. Indeed, CSC exposed fibroblast sowed repressed BCR gene expression. This gene was associated with chronic myeloid leukemia (CML) due to an active tyrosine kinase, BCR-ABL1 (41). Inhibition of BCR-ABL1 with tyrosine kinase

inhibitors or small interfering RNA was reported to be an efficiently targeted therapy for CML (42). Thus reducing BCR in CSC exposed fibroblast may suggest that the effect of CSC promoting cell proliferation do not involve BCR gene and related proteins. Cell proliferation is under the control of several specific genes such as TGFB gene. This encodes a TGFβ protein family including TGFb1 (43). Specifically, this cytokine regulates fibroblast proliferation, differentiation, adhesion, migration (43). Its inhibition following cell exposure to CSC seems to contradictory to the cell proliferation effect we demonstrated with BrdU staining and to some reported studies demonstrating the implication of TGFb1 promoting cell growth (44- 46). However, TGFβ-1 has been shown to be multifaceted, inhibiting epithelial cell cycle progression and promote apoptosis that together significantly contribute to the tumor suppressive role during carcinoma initiation and progression (47-49). Thus inhibiting the *TGFβ* gene expression by our CSC exposed fibroblast supports the proliferative phenotype we demonstrated in this study. Multiple exposures of the cells to CSC for long time (10 days) demonstrated a reduced CASP8 gene expression. CASP8 gene is well known playing active role in mediating cell apoptosis (50). Its inhibition following fibroblast exposure to CSC confirmed the inactivation of apoptotic cell signaling pathway and but the activation of those (CKD4 and V-SRC) involved in cell proliferation. Altogether, gene profiling supported the effects of CSC promoting fibroblast proliferation. However, further studies addressing the mechanisms of such effect should be performed in the future to shed light on the consequence of such fibroblast proliferation on human oral health of smokers. One of the possibilities to support this gene profiling result was to investigate the telomere length and the telomerase activity. Our results demonstrated a telomere shortening and an increased telomerase activity, when the cells were repeatedly exposed to 10% of CSC. In human cell, telomerase plays a key role maintaining the telomere length (51). Telomerase was reported to be essential for the

long-term proliferation potential of stem cells and cancer cells, and for normal tissue renewal (52). Since CSC-exposed fibroblast showed proliferative phenotype, this is supportive of the high level of telomerase activity by these CSC exposed cells to maintain telomere length preventing cell death (53). Further studies are needed to confirm such hypothesis.

3.6 Conclusion

Repeated exposure of primary human gingival fibroblasts to cigarette smoke condensate for 10 and 20 days lead to the selection of a proliferative subpopulation of fibroblasts. Through gene profiling analyses, we demonstrate the activation of certain gene involved in maintaining the growth/proliferation the selected genes include *SRC* and *CDK4* genes. Furthermore, CSC-exposed fibroblast expressed a high level of telomerase activity with telomere shortening at high doses of CSC.

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3.8 Contributions of the authors: Conceived and designed the experiments: MR, AZ, WC and AS. Performed the experiments: AA, EJ, AS and MR. Analyzed the data: AA, EJ and MR. Manuscript preparation: AA, EJ, AS, AZ, WC and MR.

3.9 Conflict of interests: The authors declare that they do not have any conflict of interests.

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Fig. 1

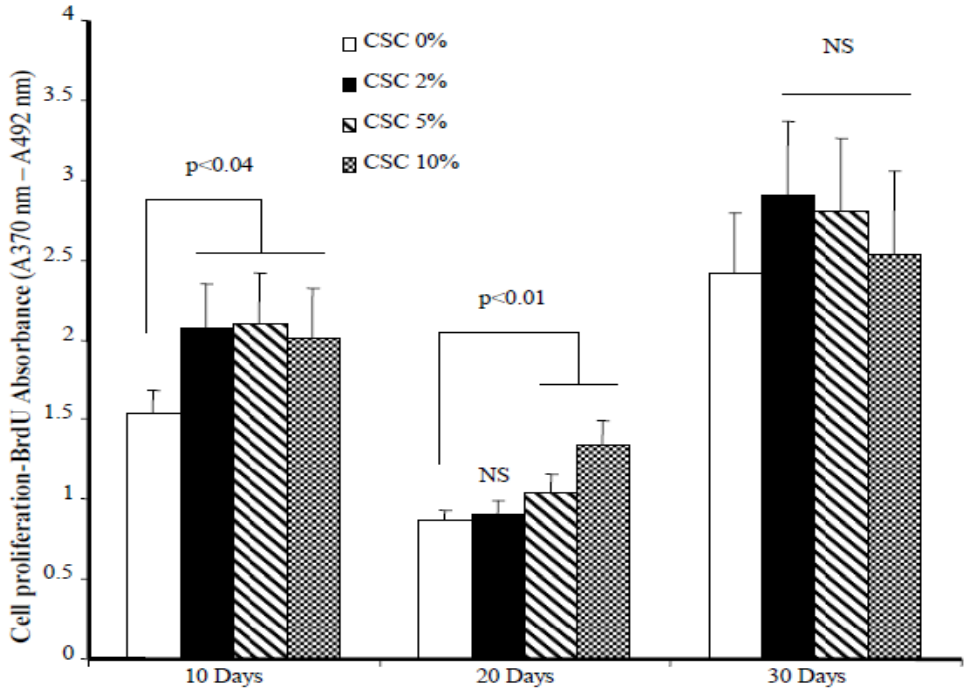

Figure 3-1: Cigarette smoke condensate increased gingival fibroblast proliferation: Cells were seeded on culture plates and allowed to adhere and proliferate till they reach 50% confluence. Cultures were then exposed to CSC 3 time a day for 10, 20 or 30 days. Cell proliferation was assessed by using BrdU/anti-BrdU assay; (n = 5).

Fig. 2

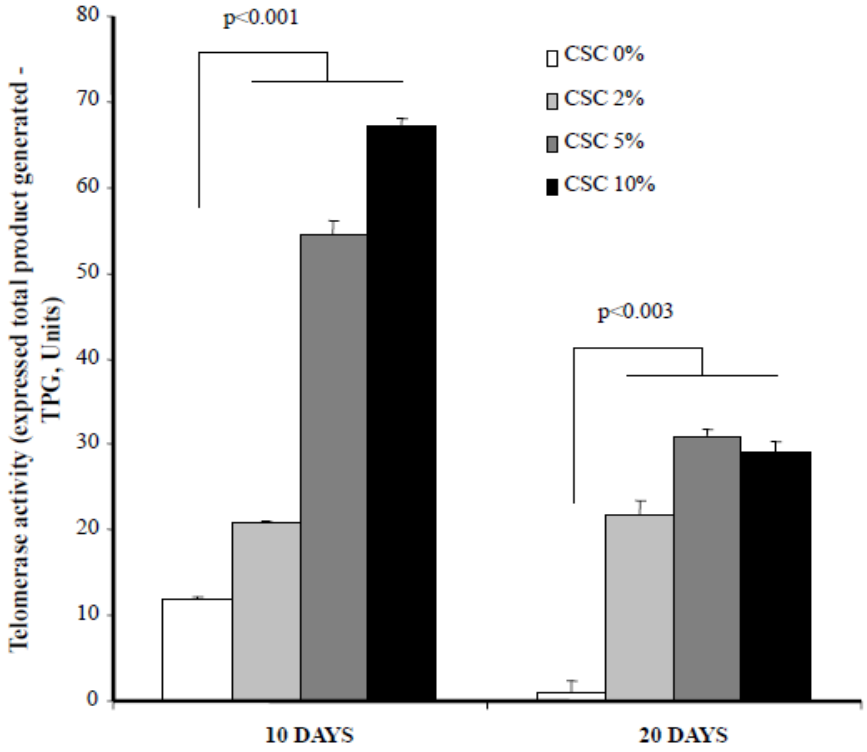

Figure 3-2: Cigarette smoke condensate exposure increased telomerase activity in gingival human fibroblasts: Following exposure or not to CSC, cells were used to extract cell lysates. Extracted proteins were subjected to a Telomeric Repeat Amplification Protocol (TRAP) using primer mixes and iTaq polymerase (BioRad) to amplify telomeric repeats. The relative telomerase activity of each extract was determined by taking the ratio of the entire telomerase ladder to that of the internal control, (n = 5). Experiments were controlled with a Primer-dimer/PCR negative control and a Telomerase-positive control included in the kit.

Fig. 3

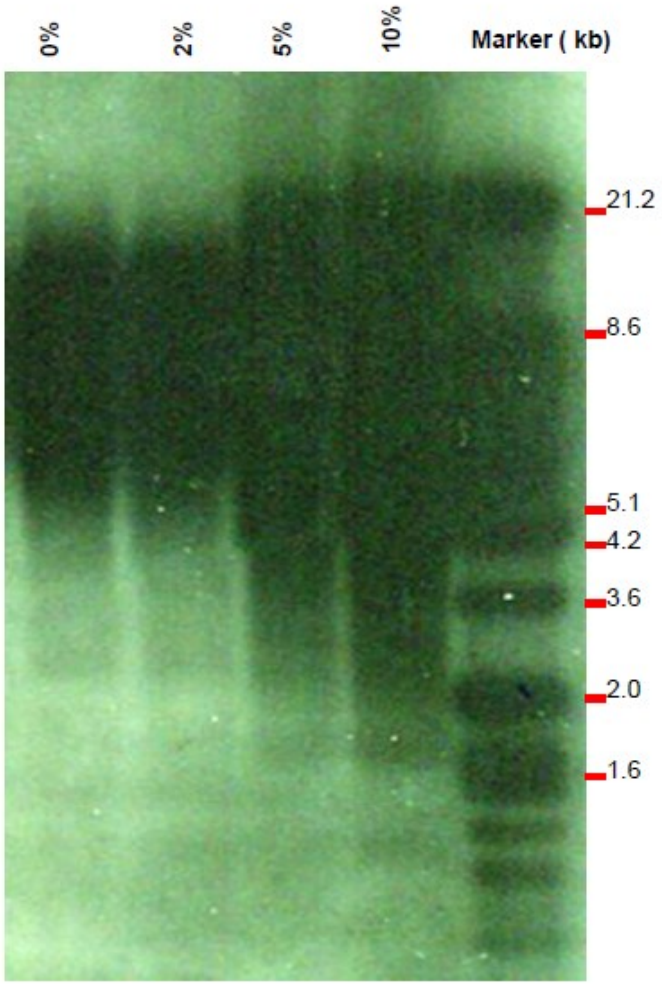

Figure 3-3: Telomere length analyses. After the exposure to CSC gingival fibroblasts were used to extract total DNA. The average telomere length was measured using the Telo-TAGGG Telomere Length assay. DNA was digested using a Hinf I / Rsa I restriction enzyme mixture then migrated on 0.8% agarose gel. Following southern transfer of digested DNA on nitrocellulose membranes, these were incubated with Anti-DIG-AP antibodies for 30 minutes. Detection was performed with chemiluminescence reaction and visualized on X-ray films and using a Chemicon Bioimaging system.

Table 3-1: List of oncogenes & tumor suppressor genes detected by PCR array (PAHS-502Z): Functional gene grouping

Pathway	Genes
Oncogenes	*BAX, BCL2L1, CASP8, CDK4, ELK1, ETS1, HGF, JAK2, JUNB, JUND, KIT, KITLG, MCL1, MET, MOS, MYB, NFKBIA, NRAS, PIK3CA, PML, PRKCA, RAF1, RARA, REL, ROS1, RUNX1, SRC, STAT3, ZHX2*
Tumor Suppressor Genes	*ATM, BRCA1, BRCA2, CDH1, CDKN2B, CDKN3, E2F1, FHIT, FOXD3, HIC1, IGF2R, MEN1, MGMT, MLH1, NF1, NF2, RASSF1, RUNX3, S100A4, SERPINB5, SMAD4, STK11, TP73, TSC1, VHL, WT1, WWOX, XRCC1*
Both Oncogenic & Tumor Suppressor Properties	*BCR, EGF, ERBB2, ESR1, FOS, HRAS, JUN, KRAS, MDM2, MYC, MYCN, NFKB1, PIK3C2A, RB1, RET, SH3PXD2A, TGFB1, TNF, TP53*
Transcription Factors	*ABL1, BRCA1, BRCA2, CDKN2A, CTNNB1, E2F1, ELK1, ESR1, ETS1, FOS, FOXD3, HIC1, JUN, JUNB, JUND, MDM2, MEN1, MYB, MYC, MYCN, NF1, NFKB1, PML, RARA, RB1, REL, RUNX1, RUNX3, SMAD4, STAT3, TGFB1, TNF, TP53, TP73, TSC1, VHL, WT1, ZHX2*
Epithelial-to-Mesenchymal Transition	*BRCA2, CDKN2B, CTNNB1, ERBB2, HGF, JAK2, KIT, MCL1, NF1, RUNX3, S100A4, SMAD4, TGFB1, VHL*
Angiogenesis	*AKT1, CTNNB1, EGF, ERBB2, NF1, PML, RUNX1, TGFB1*
Apoptosis	*BAX, BCL2, BCL2L1, BRCA1, CASP8, E2F1, MCL1, MGMT, TNF, VHL*
Cell Adhesion	*APC, CDH1, CDKN2A, CTNNB1, KITLG, NF1, NF2, TGFB1*
Cell Cycle	*ATM, BRCA1, BRCA2, CCND1, CDK4, CDKN1A, CDKN2A, CDKN2B, CDKN3, E2F1, HGF, MEN1, STK11, TP53*

Table 3-2: Oncogenes and tumor suppressor genes modulated by cigarette smoke condensate

Pathway	Gene	Description	Fold Change	
			10 days	20 days
Oncogenes	*CASP8*	Caspase 8, apoptosis-related cysteine peptidase	-2.5	ND
	REL	V-rel reticuloendotheliosis viral oncogene homolog (avian)	-1.9	ND
	JUND	Jun D proto-oncogene	-1.8	1.7
	CDK4	Cyclin-dependent kinase 4	1.6	ND
	SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	9.9	-1.9
	RARA	Retinoic acid receptor, alpha	ND	-11.4
	NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	ND	-2.7
	RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	ND	-2.4
	ETS1	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	ND	-2
	MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	ND	-2
	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	ND	1.6
	PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	ND	1.7
Tumor Suppressor Genes	*BRCA2*	Breast cancer 2, early onset	-1.9	ND
	TSC1	Tuberous sclerosis 1	-1.9	ND
	WWOX	WW domain containing oxidoreductase	-1.6	-2
	BRCA1	Breast cancer 1, early onset	ND	-2
	HIC1	Hypermethylated in cancer 1	ND	-1.7
	CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	ND	-1.6
	MGMT	O-6-methylguanine-DNA methyltransferase	ND	1.7

Table 3-3: Both onco/tumor suppressor and transcription factor genes modulated by cigarette smoke condensate.

Pathway	Gene	Description	Fold Change	
			10 days	20 days
Both Oncogenes and Tumor suppressor genes	*MYCN*	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	-5	ND
	HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	-1.7	ND
	BCR	Breakpoint cluster region	-1.5	-1.7
	ESR1	Estrogen receptor 1	1.5	-157
	TGFB1	Transforming growth factor, beta 1	ND	-1.8
	KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	ND	-1.6
Transcription Factors	*PML*	Promyelocytic leukemia	-1.6	ND
	JUN	Jun proto-oncogene	-1.6	-2
	RB1	Retinoblastoma 1	1.6	-1.8
	RARA	Retinoic acid receptor, alpha	ND	-11
	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	ND	-2
	NF1	Neurofibromin 1	ND	-1.8

Table 3-4: Apoptotic, cell adhesion molecules and cell cycle genes modulated by cigarette smoke condensate.

Pathway	Gene	Description	Fold Change	
			10 days	20 days
Apoptosis	BCL2	B-cell CLL/lymphoma 2	-2	-1.6
	CASP8	Caspase 8, apoptosis-related cysteine peptidase	-3	ND
	BRCA1	Breast cancer 1, early onset	ND	-2
	MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	ND	-2
	MGMT	O-6-methylguanine-DNA methyltransferase	ND	1.7
Cell adhesion molecules	APC	Adenomatous polyposis coli	1.6	1.6
	NF1	Neurofibromin 1	ND	-1.8
	TGFB1	Transforming growth factor, beta 1	ND	-1.8
Cell Cycle	BRCA2	Breast cancer 2, early onset	-1.9	ND
	CCND1	Cyclin D1	ND	-2
	BRCA1	Breast cancer 1, early onset	ND	-2
	TP53	Tumor protein p53	ND	-1.6
	CDK4	Cyclin-dependent kinase 4	1.6	ND
	CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	ND	-1.6

Chapter 4

Discussion

4.1 Discussion

In this study, we investigated two primary objectives:

1. To study the effect of a chronic exposure to high doses of CSC and
2. To study the effect of a short but repeated exposure to low doses of CSC on fibroblast's viability and apoptosis.

We demonstrated that the continuous exposure of normal human gingival fibroblasts to high concentrations of cigarette smoke condensate resulted in a change of fibroblast's morphology and shape and low viability as detected by optical microscope observation and MTT assay. The effect of cigarette smoke on human gingival fibroblasts may occur through apoptotic and necrotic mechanisms. Indeed, fibroblasts exposed to CSC showed a significant increase in the number of apoptotic/necrotic cells, as ascertained by annexin-V/propidium iodide staining. Cigarette smoke condensate therefore had a significant effect on the gingival fibroblasts by reducing the number of viable cells. These results support those previously reported in other models, including human bronchial (47) and nasal epithelial cells (48).

Apoptosis is crucial to maintaining normal tissue homeostasis. Increasing evidence shows that disturbing the balance between apoptosis and proliferation contributes to pulmonary disease (49) and controls different cancer type's development. The increase of apoptosis in emphysema patients was paralleled by an increased expression of Bax proteins (50). Our PCR Array data demonstrate that CSC modulated the expression by gingival fibroblasts of several genes involved in apoptotic/necrotic pathways including an increase in Bax mRNA expression. We suggest that the Bax upregulation is involved in the modulation of fibroblast apoptosis through the disruption of the pro-apoptotic–anti-apoptotic balance in the mitochondrial apoptosis pathway (49). Furthermore, through an opening of permeability transition pores, the activation and translocation

of Bax to the mitochondria induces a release of apoptotic -inducing proteins into the cytosol, which ultimately executes the apoptosis (49). Apoptosis onset is associated with the proteolytic activation of caspases, which play a critical role in triggering apoptosis (51). Caspases are synthesized as proenzymes that are processed by self-proteolysis and/or cleavage by another protease to their active forms in cells undergoing apoptosis (52). Caspase-3, a major trigger of apoptosis, is promoted during the early apoptotic stage, and the activated form is a marker for cells undergoing apoptosis (53). In this study, the gingival fibroblasts exhibited significant caspase-3 activity after chronic exposure to CSC 5 days.

In promoting gingival fibroblasts cell apoptosis/necrosis, CSC may hamper cell growth and promote apoptosis through specific genes such as *p53*. P53 protein is one of the most important intracellular sensors for damage signals and stress (e.g., DNA damage) (54-56). P53 can initiate apoptosis by activating signaling pathways that will activate mitochondrial apoptotic processes. It can induce mitochondrial dysfunction and the loss of mitochondrial outer membrane permeabilization and membrane potential through activation of pro-apoptotic proteins. This is supportive of our study which shows that CSC increased p35 gene expression and protein production by gingival fibroblasts. These are in accordance with previous studies reporting P53 increasing in cigarette smoke-exposed endothelial cells (57), oral epithelial cells (4), lung epithelial cells (58) and fibroblasts (59). Future studies using normal human gingival cells integrated into an engineered connective tissue will no doubt enable us to learn more regarding the cellular and molecular pathways involved in the interaction between whole cigarette smoke and human tissues and disease development.

In summary of our first objective, we demonstrated that exposure to CSC significantly reduced normal human gingival fibroblast growth through an apoptosis/necrosis pathway involving Bax and p53 increased expression and increased caspase-3 activity. This negative impact of smoke on

gingival fibroblast may compromise the interaction between epithelial cells and fibroblasts leading to tissue dysfunction. Further studies related to the mechanism of action of cigarette smoke are thus necessary to shed light on the connection between cigarette smoke, fibroblast dysfunction and oral diseases such as periodontitis and cancer.

To complete the second objective, we investigate the effect of a short but repeated exposure to low concentrations of CSC on gingival fibroblast behavior. When exposed to low but repeated doses of CSC for a longer period. Gingival fibroblasts seemed to respond differently depending on CSC concentration and the exposure regime. We demonstrated that CSC exposed fibroblast showed increased cell division. We noticed it led to a significant increase in the number of cells, in the 10 days CSC stressed cultures. One explanation for this proliferation following a repeated exposure to low concentrations of CSC is probably due to cell cycle acceleration and also to the telomerase activity, which are known as triggering factors for cancer development. Our hypothesis is that a repeated exposure to cigarette can induce molecular changes (mutation, methylation and gene expression changes) disturbing the balance between apoptosis, proliferation and in oncogenesis. This is supported by our PCR array data demonstrating that CSC modulated the expression of different oncogenes, apoptosis-related genes, Tumor Suppressor Genes and Transcription Factors genes in gingival fibroblasts. This particular treatment of CSC significantly increased gene expression of SRC (oncogene), Cyclin-dependent kinase 4 (CDK-4) and Retinoblastoma 1, and significant a down-regulated MYCN, CASP8 and BCL2 genes after 10 days of 10% CSC exposure compared to non-stimulated cells. This is in accordance with previous studies which have reported a similar increase (60).

One of the factors leading to oral cancer is uncontrolled cell proliferation which is controlled by telomerase activity. Telomerase is reactivated in 80-90% of cancers, plays an important role in the

maintenance of telomere ends, and telomerase activity is known to be higher in the cancer cells than of normal cell, suggesting anti-telomerase drugs as therapies controlling cancer development (61, 62). Previous studies have shown that smoking is associated with increased telomerase activity (61, 62). In our work, we have shown that gingival fibroblasts express significantly higher telomerase activity after treatment with low repeated exposures of CSC. This increase in telomerase activity did not correlate with an increase in average telomere length. However, treatment with CSC did alter the range of telomere lengths when compared to controls. CSC-exposed cells had longer and shorter telomeres than controls.

In summary, we demonstrated that the effect of a short but repeated exposure to CSC significantly modulates human gingival fibroblast response. A repeated CSC exposed increase cell division may be due to cell cycle changes, increase the oncogene genes expression, significantly down-regulate apoptosis genes such as CASP8 and BCL2 genes and increase the telomerase activity. These are the principal oral cancer triggers factors. Further studies are needed to demonstrate the link between epigenetic modifications on apoptosis genes down-regulated by cigarette and their association with oral cancer development

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